

Remarks

Claims 44-47 were pending. By this amendment, no claims are cancelled or added. Therefore, claims 44-47 are still pending.

Claims 44, 46, and 47 are amended to clarify the claims.

The specification is amended to recite the parent number of the parent application, and to amend the abstract in view of the claims being prosecuted.

No new matter is introduced by this amendment, and no amendments are made to distinguish prior art.

Summary of Telephone Interview with the Examiner

Applicants thank Examiner Para for the courtesy of a telephone interview with Applicant's Representative Sheree Lynn Rybak, Ph.D. on March 1, 2007. During this interview the outstanding 35 U.S.C. §§ 112, first paragraph and 102(b) rejections were discussed. Agreement was not reached.

Examiner Para explained that the claims were rejected under 35 U.S.C. § 112, first paragraph, because the specification does not describe how to isolate the endophytes, and no deposit of endophytes has been made. Applicant's representative explained that methods of isolating endophytes are routine, and thus there is no duty to make such a disclosure in the specification. In addition, Applicant's representative noted that Applicant would be willing to make a deposit of endophyte as it is Applicant's understanding that such a deposit would not constitute new matter (see MPEP 2406.02), if this would overcome the outstanding rejection. Examiner Para recommended filing a CIP application.

With regards to the 35 U.S.C. § 102(b) rejection, Applicant's representative noted that the units in the cited Siegel *et al.* document are in ppm (parts per million), not ppb (parts per billion). The claims recite ppb, not ppm. Therefore, Siegel *et al.* does not anticipate the claims. Examiner Para agreed to reconsider Siegel *et al.* in view of this information.

Objections to the Specification

The abstract was objected to on the ground that it is not drawn to the claimed invention. The abstract has been amended to clarify that the application is directed to endophytes.

The specification was objected to on the ground that page 14 of the specification recites that the seeds had about 183 ppb of ergovaline, while claim 47 recited about 55 ppb of ergovaline. Claim 47 has been amended to clarify that the level of ergovaline in seeds is about 183 ppb.

In view of these amendments, Applicant requests that the objections to the specification be withdrawn.

35 U.S.C. § 101

Claims 44-47 are rejected as being directed to non-statutory subject matter. Applicant requests reconsideration.

Claims 44 and 46 are amended to recite that the endophytes are isolated.

In view of this amendment, Applicant requests that the 35 U.S.C. § 101 rejection be withdrawn.

35 U.S.C. § 112, written description

Claims 44-47 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. Applicant disagrees and requests reconsideration.

It is asserted in the Office action that the claims are drawn to any endophyte of a tall fescue. This is incorrect. The claims are directed to endophytes isolated from a tall fescue grasses grown from a particular ATCC seed deposit (ATCC PTA-3825) (claims 44-45) or endophytes isolated from the deposited seed itself (claims 46-47).

It is asserted the Applicant did not isolate the claimed endophyte. It is not relevant whether the inventor actually reduced to practice the claimed invention (for example by isolating endophytes from the plant or seed), or constructively reduced to practice (for example by providing sufficient information for one skilled in the art to reduce to practice the claimed invention) (see MPEP 2406.02).

Applicant disclosed the seed from which the endophyte can be isolated or which can be used to grow grass from which endophytes can be isolated. Methods of isolating endophytes from seed or grass plants were routine in the art as of the priority date of the present application

(January 30, 2002). For example, Bony *et al.* (*New Phytologist* 152:125-37, 2001; Exhibit A), describe a method of isolating endophytes from germinated grass seed (see page 126, last two paragraphs). Siegel *et al.* (*Annu. Rev. Phytopathol.* 25:293-315, 1987; Exhibit B) describe a method of isolating endophytes from grass plants (see pages 297-298), as do Latch *et al.* (*Mycotaxon*, XX:535-50, 1984; Exhibit C, see page 536). As stated in MPEP 2164.05(a), "The specification need not disclose what is well-known to those skilled in the art and preferably omits that which is well-known to those skilled and already available to the public." Therefore, as the seed from which the endophyte can be isolated (or can be used to grow a plant from which the endophyte can be isolated) is provided under the terms of the Budapest Treaty, and methods for isolating endophytes are well-known to those skilled in the art, the specification provides ample written description for the claimed isolated endophytes.

Therefore, Applicant requests that the 35 U.S.C. § 112, first paragraph written description rejection be withdrawn.

35 U.S.C. § 112, enablement

Claims 44-47 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. Applicant disagrees and requests reconsideration.

Although the claimed endophytes have not yet been deposited under the terms of the Budapest Treaty, seeds from which the endophytes can be isolated are deposited under the terms of the Budapest Treaty (ATCC PTA-3825). As described above, one skilled in the art can isolate endophyte from such seed using routine methods. Therefore, the claims are enabled, even in the absence of a biological deposit of the endophytes themselves.

However, Applicant is willing to make a deposit of the claimed endophyte if this would expedite prosecution. Unfortunately, Examiner Para stated during the telephone interview with Applicant's representative that making a biological deposit of the claimed endophyte at this time would constitute new matter. Applicant disagrees and requests reconsideration in view of MPEP 2406.02, which states "the rules will not preclude such a situation as there is no requirement in the patent law that an actual reduction to practice occur as a condition precedent to filing a patent application." This section of the MPEP states that it is acceptable to make an original deposit after the effective filing date of an application for patent. In such a case, "applicant is required to promptly submit a statement from a person in a position to corroborate that the biological

material which is deposited is a biological material specifically identified in the application (the filing date of which is relied upon) as filed.” Applicant is willing to make such a statement in combination with a biological deposit to expedite prosecution.

If the Office agrees with Applicant’s argument, Applicant is willing to make a deposit under the terms of the Budapest Treaty and make the statement required under MPEP 2406.02, and will amend the specification accordingly.

Therefore, Applicant requests that the 35 U.S.C. § 112, first paragraph enablement rejection be withdrawn.

35 U.S.C. § 102(b)

Claims 44-47 are rejected under 35 U.S.C. § 102(b) as anticipated by Siegel *et al.* (*J. Chem. Ecol.* 16:3301, 1990). Applicant disagrees and requests reconsideration.

It is asserted on page 7 of the Office action that Siegel *et al.* teach a tall fescue having endophyte and 44 ppb of lolitrem B and 1.3 ppb of ergovaline (Table 2, plant 12), and thus anticipate the claims. This assertion is incorrect, as the units in Table 2 are ppm (parts per million) not ppb (parts per billion). Therefore, plant 12 disclosed in Siegel *et al.* has significantly more lolitrem B and ergovaline than the claimed endophytes.

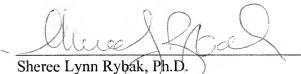
Because Siegel *et al.* do not anticipate the claims, Applicant requests that the 35 U.S.C. § 102(b) rejection be withdrawn.

If there are any minor issues to be resolved before a Notice of Allowance is issued, the Examiner is invited to telephone the undersigned.

Respectfully submitted,

KLARQUIST SPARKMAN, LLP

One World Trade Center, Suite 1600
121 S.W. Salmon Street
Portland, Oregon 97204
Telephone: (503) 595-5300
Facsimile: (503) 595-5301

By 
Sheree Lynn Rybak, Ph.D.
Registration No. 47,913

The relationship between mycotoxin synthesis and isolate morphology in fungal endophytes of *Lolium perenne*

Sylvie Bony¹, Nathalie Pichon², Catherine Ravé¹, Andrée Duris¹, François Balloumet² and Jean-Jacques Guillaumin²

¹Centre INRA de Clermont-Ferrand/Thiers/Lyon, UMR 188, Toxicologie et Métabolisme Comparés des Xenobiotiques, Ecole Nationale Vétérinaire de Lyon, BP 83, 69280 Marcy l'Etoile, France; ²Centre INRA de Clermont-Ferrand/Thiers/Lyon, UMR Amélioration et Santé des Plantes, 234 Avenue du Brezet, 63099 Clermont-Ferrand cedex, France

Author for correspondence:
Jean-Jacques Guillaumin
Tel +33 4 7362 4440
Fax +33 4 7362 4459
Email guillaumin@clermont.inra.fr

Received: 15 March 2001
Accepted: 14 June 2001

Summary

- Variability in the fungal endophytes of 83 natural populations of *Lolium perenne* (perennial ryegrass) from Europe was assessed
- One plant per population was used for endophyte isolation and mycotoxin analysis. Variability in three isozymes, colony morphology and growth rate on potato dextrose agar (PDA), and synthesis of ergovaline, lolitrem B and peramine was recorded.
- Three species were found among 94 strains isolated: *Neotyphodium lolii*, *Neotyphodium* sp. (LpTG-2) and *Gliocladium*-like. The most frequent species was *N. lolii*, which showed high variability. In 12 populations, a single plant harboured two different endophytes. One-third of the isolates of *N. lolii* did not produce ergovaline whereas a few isolates did not produce lolitrem B. Ergovaline and lolitrem B-deficient strains, but not the few peramine-deficient isolates, had characteristic morphologies on PDA. No isolate was deficient for both ergovaline and lolitrem B synthesis
- Selection of ergovaline and lolitrem-deficient strains based only on the morphology of the isolates in culture may be possible.

Key words: fungal endophytes, *Lolium perenne* (perennial ryegrass), *Neotyphodium*, intraspecific fungal variability, ergovaline, lolitrem B, peramine.

© New Phytologist (2001) 152: 125–137

Introduction

The presence of fungal endophytes in the seeds of *Lolium perenne* L. was first observed by Guérin (1898). McLennan (1920) studied the distribution and development of the mycelium in the different organs of the grass. Sampson (1937) was the first to isolate an endophytic fungus from *L. perenne* and to grow it in pure culture.

In New Zealand, a damaging tremorgenic disease of sheep and cattle, 'ryegrass staggers', had long been reported and attributed to the consumption of perennial ryegrass by grazing animals (Cunningham & Hartley, 1959). Later on, ryegrass staggers was shown to be caused by alkaloids (Aasen *et al.*, 1969) and the implication of the fungal endophyte in the toxicosis was eventually established by Fletcher & Harvey (1981).

Sampson (1933) observed the presence of two different endophytic fungi in *Lolium perenne* and Latch *et al.* (1984)

isolated them. The first type was thin, poorly stainable and highly branched. It was generally sterile in culture, but the production of a few conidiophores resembling *Gliocladium* led to this species being called 'Gliocladium-like' (Philipson, 1989).

The second, more frequent, type consisted of twisted, poorly branched hyphae. It was shown to belong to clavicipitaceae and described as a new species, *Acremonium loliae* Latch, Christensen and Samuels (later changed to *Acremonium lolii*). Within the genus *Acremonium*, this species was classified in a section *Albo-lanosa* created by Morgan-Jones & Gams (1982) to include the anamorphs of the clavicipitaceae. However, the fungi belonging to this section were reclassified by Glenn *et al.* (1996) into the new genus *Neotyphodium*, the clavicipitaceous endophytes of *L. perenne* being named *N. lolii* (Latch, Christensen & Samuels) Glenn, Bacon & Hanlin.

Christensen *et al.* (1991, 1993) introduced an additional distinction among the clavicipitaceous endophytes of perennial

regrass on the basis of growth rate, isolation delay, sporulation at 25°C and isozyme pattern. The majority of isolates could be accommodated as *Neotyphodium lolii*. However, a few isolates showing higher growth were just named by the abbreviation IpTG-2.

The clavicipitaceous endophytes of perennial ryegrass are known to synthesize several mycotoxins, among which three are particularly important: lolitrem B, a hemiterpene molecule responsible for 'ryegrass staggers'; ergovaline, a compound belonging to the ergoserpine group, which has vasoconstrictor effects and causes various diseases on grazing mammals ('fescue-foot' and 'fescue toxicosis'); and peramine, a tripeptide which is repellent and toxic for insects but not for mammals. The strains of IpTG-2 which have been studied synthesized ergovaline and peramine, but not lolitrem B, while variation was observed among strains of *N. lolii* for the synthesis of lolitrem B, ergovaline and peramine (Christensen *et al.*, 1993). Schardl *et al.* (1994) showed that IpTG-2 is a heteroploid species originating from interspecific hybridization between *Neotyphodium lolii* and the parasitic species *Epichloe typhina*.

The dual action of clavicipitaceous endophytes (beneficial effects on the host vs risk of toxicity for grazing animals) sets forage grass breeders special problems. Sometimes, these have been solved by removing the endophyte from the cultivars selected for pasture and by maintaining or introducing it into varieties selected for turf. Another strategy, developed mainly in New Zealand (Latch, 1989), proposed to select harmless clavicipitaceous endophytes (producing no or little ergovaline or lolitrem) and to inoculate these strains to commercial cultivars. This strategy involves an extensive study of the variability of the endophytes of *Lolium perenne*. Such research was conducted on a large scale in New Zealand (Latch, 1994; Fletcher & Easton, 1997).

Very few studies have been conducted on this subject in Europe, despite the fact that *Lolium perenne* probably originated from the Near East and has diversified in Europe (Balfourier *et al.*, 2000). Maximum genetic variability would therefore be expected in Europe.

In France, 547 natural populations of *Lolium perenne* were collected from 1983 to 84 (Charmet *et al.*, 1990, 1993; Balfourier & Charmet, 1991). About half of these populations were sampled at random and checked for the presence of endophytes (Lewis *et al.*, 1997; Ravel, 1997). One, or several, endophytes were found in 188 populations out of 262 (72%). The present study was conducted on part of this material. The objectives were to identify the fungal species involved and to describe intraspecific variability, especially as concerns the production of mycotoxins.

Materials and Methods

Material

Seventy-three populations with endophytes were selected from the populations studied by Ravel (1997). They were

conserved at INRA Clermont-Ferrand as seeds and/or living plants (Table 1a). The material was selected on geographical and ecological bases to achieve homogeneous cover of the country, and to include a wide range of ecological situations.

For comparison, 10 populations of European, non-French origin, were included in the study (Table 1b). In addition, six standard isolates (four of *Neotyphodium lolii* and two of species IpTG-2) were obtained from G. C. M. Latch from AgResearch, New Zealand (Table 1c). These last isolates originated from natural populations in Spain and Southern France.

Methods

General procedure

For each population, one plant had been selected in the field, and the presence of an endophyte had been verified. The 83 populations were represented by seeds originating from only one plant per population.

Isolations were carried out from the 83 seed lots. All the isolates were grown on PDA (Potato Dextrose Agar, Sigma Chemical, 39 g l⁻¹) to observe their macroscopical morphology and measure their linear growth. Mycelium grown in liquid culture was checked for the isoforms of three enzymes, to allow the taxonomical identification of the isolates.

Straws from the 83 plants from which the seeds had been taken had been harvested in early August, frozen and lyophilized, and the analysis of ergovaline, lolitrem B and peramine was carried out on this material.

Isolation

Seeds were sterilized for 4 h in commercial bleach (12%Cl₂), then rinsed with sterile water and sterilized again for 3 min in Bayrochlor® (from Bayrochlor Company). They were rinsed three times with sterile water and the glumellas were removed. The seeds were air-dried and then sown in Petri dishes containing PDA plus penicillin and streptomycin (100 µg g⁻¹ each). Approximately 10 seeds were placed in each dish. The Petri dishes were sealed with adhesive tape, placed on their side and incubated in the dark at 23°C, the seeds, slightly sunk in agar, were aligned horizontally, with their coleoptiles upwards (Fig. 1).

Every 2 d the seeds were observed for germination and the appearance of mycelium on the medium. Under these conditions, the fungus was observed to grow from the cotyledon, the coleoptile and even the seminal roots. In the majority of cases, the coleoptile was cut into pieces with a microscalpel and the pieces subcultured on a new PDA medium. The fungi obtained were subcultured, paying particular attention to the appearance of any morphologically different colonies from the same seed lot: colonies showing a clearly different aspect or growth rate were subcultured separately.

Table 1 Taxonomical identification, morphology and growth rate of the isolates. (a) Populations of French origin; (b) Populations of non-French origin; (c) Reference isolates

| (a) | (b) | (c) | Fungal species ^a | MG ^b | Linear growth ^c |
|-----------|----------|-----|-----------------------------|-----------------|----------------------------|
| n° popul. | isolates | | | | |
| 10119 | 10119 | | <i>Nicotophodium lolu</i> | III | C7 |
| 10151 | 10151 | | <i>N. lolu</i> | III | C6 |
| 10156 | 10156 | | <i>N. lolu</i> | III | C6 |
| 10170 | 10170 | | <i>N. lolu</i> | III | C6 |
| 10180 | 10180 | | <i>N. lolu</i> | II | C7 |
| 10184 | 10184 | | <i>N. lolu</i> | III | C4 |
| 10208 | 10208 | | <i>N. lolu</i> | I | C9 |
| 10224 | 10224 | | <i>N. lolu</i> | I | C9 |
| 10251 | 10251 | | <i>N. lolu</i> | II | C7 |
| 10256 | 10256 A | | <i>N. lolu</i> | III | C6 |
| | 10256 B | | <i>N. lolu</i> | IV | C2 |
| 10261 | 10261 | | <i>N. lolu</i> | III | C5 |
| 10266 | 10266 | | <i>N. lolu</i> | II | C4 |
| 10304 | 10304 | | <i>N. lolu</i> | III | C6 |
| 10319 | 10319 | | <i>N. lolu</i> | III | C6 |
| 10359 | 10359 | | <i>N. lolu</i> | III | C6 |
| 10361 | 10361 A | | <i>N. lolu</i> | VI | C7 |
| | 10361 B | | <i>N. lolu</i> | II | C8 |
| 10363 | 10363 | | <i>Gliocladium-like</i> | VIII | C4 |
| 10364 | 10364 | | <i>N. lolu</i> | II | C7 |
| 10365 | 10365 | | <i>N. lolu</i> | II | C6 |
| 10367 | 10367 | | <i>N. lolu</i> | II | C8 |
| 10370 | 10370 A | | <i>N. lolu</i> | II | C8 |
| | 10370 B | | <i>N. lolu</i> | III | C6 |
| 10405 | 10405 | | <i>N. lolu</i> | II | C7 |
| 10452 | 10452 | | <i>N. lolu</i> | III | C6 |
| 10458 | 10458 | | <i>N. lolu</i> | III | C5 |
| 10505 | 10505 | | <i>N. lolu</i> | II | C6 |
| 10509 | 10509 A | | <i>N. lolu</i> | II | C6 |
| | 10509 B | | <i>N. lolu</i> | VI | C5 |
| 10515 | 10515 A | | <i>N. lolu</i> | V | C2 |
| | 10515 B | | <i>N. lolu</i> | II | C7 |
| 10520 | 10520 | | <i>N. lolu</i> | II | C6 |
| 10551 | 10551 | | <i>N. lolu</i> | III | C6 |
| 10557 | 10557 | | <i>N. lolu</i> | II | C7 |
| 10573 | 10573 | | <i>N. lolu</i> | III | C6 |
| 10606 | 10606 | | <i>N. lolu</i> | II | C6 |
| 10611 | 10611 | | <i>N. lolu</i> | III | C6 |
| 10621 | 10621 | | <i>N. lolu</i> | II | C6 |
| 10626 | 10626 | | <i>N. lolu</i> | II | C7 |
| 10656 | 10656 | | <i>N. lolu</i> | III | C4 |
| 10667 | 10667 | | <i>N. lolu</i> | II | C6 |
| 10674 | 10674 | | <i>N. lolu</i> | III | C7 |
| 10675 | 10675 | | <i>N. lolu</i> | III | C7 |
| 10702 | 10702 | | <i>N. lolu</i> | III | C7 |
| 10707 | 10707 | | <i>N. lolu</i> | III | C5 |
| 10710 | 10710 | | <i>N. lolu</i> | III | C6 |
| 10716 | 10716 | | <i>N. lolu</i> | III | C7 |
| 10753 | 10753 | | <i>N. lolu</i> | III | C6 |
| 10763 | 10763 | | <i>N. lolu</i> | I | C9 |
| 10769 | 10769 | | <i>N. lolu</i> | III | C6 |
| 10770 | 10770 | | <i>N. lolu</i> | III | C5 |
| 10873 | 10873 | | <i>N. lolu</i> | I | C9 |
| 10880 | 10880 | | <i>N. lolu</i> | II | C7 |
| 10905 | 10905 | | <i>N. lolu</i> | I | C9 |
| 10906 | 10906 | | <i>N. lolu</i> | II | C9 |
| 10910 | 10910 | | <i>N. lolu</i> | III | C6 |
| 10917 | 10917 | | <i>N. lolu</i> | III | C6 |
| 10954 | 10954 | | <i>N. lolu</i> | II | C8 |
| 10963 | 10963 A | | <i>N. lolu</i> | IV | C1 |
| | 10963 B | | <i>N. lolu</i> | V | C4 |

Table 1 (continued)

| (a) | n° popul | isolates | Fungal species ¹ | MG ² | Linear growth ³ | |
|-----|----------|-----------------------------|-----------------------------|-----------------------------|----------------------------|----------------------------|
| | 10972 | | <i>N. lolu</i> | III | C5 | |
| | 11104 | 11104 | <i>N. lolu</i> | II | C7 | |
| | 11110 | 11110 | <i>Gliocladium-like</i> | VIII | C5 | |
| | 11115 | 11115 | <i>N. lolu</i> | II | C8 | |
| | 11118 | 11118 A | <i>N. lolu</i> | III | C6 | |
| | | 11118 B | <i>N. lolu</i> | VI | C7 | |
| | 11151 | 11151 | <i>N. lolu</i> | III | C7 | |
| | 11154 | 11154 A | <i>N. lolu</i> | III | C5 | |
| | | 11154 B | <i>Gliocladium-like</i> | VIII | C4 | |
| | 11163 | failure of isolation | | | | |
| | 11178 | 11178 | <i>N. lolu</i> | III | C9 | |
| | 11205 | 11205 A | <i>N. lolu</i> | III | C6 | |
| | | 11205 B | <i>N. lolu</i> | VI | C9 | |
| | 11214 | 11214 | <i>N. lolu</i> | III | C5 | |
| | 11264 | 11264 | <i>N. lolu</i> | III | C7 | |
| | 11274 | 11274 | <i>N. lolu</i> | II | C6 | |
| | 11280 | 11280 | <i>N. lolu</i> | III | C4 | |
| | 11302 | 11302 | <i>N. lolu</i> | III | C1 | |
| | 11314 | 11314 A | <i>LpTG-2</i> | VII | C4 | |
| | | 11314 B | <i>N. lolu</i> | III | C6 | |
| | 11315 | 11315 | <i>LpTG-2</i> | VII | C4 | |
| | 11442 | 11442 A | <i>LpTG-2</i> | VII | C3 | |
| | | 11442 B | <i>N. lolu</i> | IV | C5 | |
| (b) | | | | | | |
| | n° popul | Country | isolates | Fungal species ¹ | MG ² | Linear growth ³ |
| | 20088 | Spain | 20088 | <i>LpTG-2</i> | VII | C7 |
| | 20118 | Spain | 20118 | <i>LpTG-2</i> | VII | C4 |
| | 40128 | Italy | 40128 | <i>LpTG-2</i> | VII | C3 |
| | 40137 | Italy | 40137 A | <i>LpTG-2</i> | VII | C4 |
| | | | 40137 B | <i>N. lolu</i> | IV | C2 |
| | 70036 | Poland | 70036 | <i>N. lolu</i> | II | C6 |
| | 110005 | Yugoslavia | 110005 | <i>N. lolu</i> | V | C2 |
| | 110009 | Yugoslavia | 110009 | <i>N. lolu</i> | II | C6 |
| | 120027 | Germany | 120027 | <i>Gliocladium-like</i> | VIII | C2 |
| | 130003 | Greece | 130003 | <i>N. lolu</i> | IV | C1 |
| | 210027 | Bulgaria | 210027 | <i>N. lolu</i> | V | C3 |
| (c) | | | | | | |
| | isolates | Fungal species ¹ | MG ² | Linear growth ³ | | |
| | R 1 | <i>LpTG-2</i> | VII | C2 | | |
| | R 2 | <i>LpTG-2</i> | VII | C3 | | |
| | R 3 | <i>N. lolu</i> | VI | C9 | | |
| | R 4 | <i>N. lolu</i> | V | C4 | | |
| | R 5 | <i>N. lolu</i> | V | C3 | | |
| | R 6 | <i>N. lolu</i> | I | C8 | | |

¹Identification from patterns Malate Dehydrogenase (MDH), Phosphoglucumutase (PGM) and Phosphoglucose Isomerase (PGI). ²Morphological Groups (Fig. 2). ³Nonoverlapping groups of growth. Groups are ordered from C1, the group characterized by the highest linear growth, to C9, the group characterized by the lowest linear growth. The average growth in mm d⁻¹ is 0.353 for C1, 0.304 for C2, 0.269 for C3, 0.231 for C4, 0.193 for C5, 0.150 for C6, 0.113 for C7, 0.077 for C8 and 0.019 for C9.

Species identification by isozyme analysis

An unambiguous identification of the isolates was obtained by analysis of three isozymes: Malate Dehydrogenase (MDH),

Phosphoglucose Isomerase (PGI) and Phosphoglucumutase (PGM). The isolates were grown in V8 liquid medium containing for 1 l: V8 juice (a commercial product constituted of extracts of 8 different vegetables): 100 ml; D-glucose: 10 g;

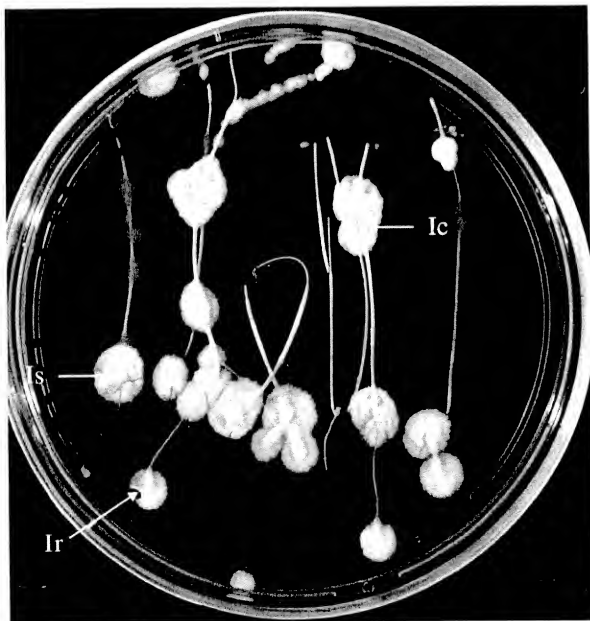


Fig. 1 Isolation of endophytes from germinating seeds of *Lolium perenne*. The fungus can be subcultured from the seed itself (Is), the coleoptile (Ic), the seminal roots (Ir).

L-asparagine: 2 g; KH_2PO_4 : 1 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$: 0.5 g; KCl: 0.25 g; FeCl_3 : 0.01 g; CaCO_3 : 2 g; chloramphenicol: 0.05 g. The pH was adjusted to 6.0. The medium was distributed into Erlenmeyer flasks (100 ml in each) and sterilized in an autoclave (110°C for 30 min). Each isolate was represented by two replicates. The flasks were subjected to gentle shaking (70 rpm) in the dark. The temperature was 18°C for those isolates which, from their morphology, appeared to be *Gliocladium*-like, and 23°C for the other isolates.

The mycelium was removed after 3 wk and lyophilized. Extraction of the enzymes, gel preparation, electrophoresis

and visualization were carried out according to the procedures described by Christensen *et al.* (1993), and Naffaa *et al.* (1998). The fungal species were identified from the description of electromorphs by the same authors.

Morphological description on PDA

Among the different morphological types which had been *a priori* subcultured from the isolations, only those that seemed stable in the course of the subsequent cultures were retained. The morphology of many isolates stabilized at the second

subculture even when variation was present in the first subculture.

These isolates were grown, observed and described in standard conditions: the culture medium was 20 ml of PDA distributed in Petri dishes 9 cm in diameter. Incubation was conducted in the dark, at $25 \pm 0.5^\circ\text{C}$. The inoculum consisted of a mycelium disk cut with a 4-mm borer from the margin of a 1-month-old colony also grown on PDA. The Petri dishes were sealed with adhesive tape. The linear growth of the isolates was measured on the same Petri dishes. The diameter of each colony was measured after 45 d from the average of two perpendicular diameters. The growth rate was evaluated in mm d^{-1} . There were at least five replicates for each isolate.

Mycotoxin analysis

Lolitrein B was analysed by an adaptation of the HPLC method of Gallagher *et al.* (1985). Ground freeze-dried straw was mixed with chloroform-methanol (2:1) for 1 h on a horizontal shaker. After centrifugation, the supernatant was filtered and evaporated under a nitrogen stream. The dry residue dissolved in dichloromethane was cleaned on a Si Bond Elut column (Varian) conditioned with dichloromethane. Lolitrein B was then eluted from the column with dichloromethane-acetonitrile (80:20). The elution solution was evaporated under a nitrogen stream and the residue was dissolved in the HPLC mobile phase. The HPLC analysis was performed using a Thermoquest liquid chromatograph system equipped with a silica column (250 mm \times 4.6 mm, 20 μl injection) and a fluorescence detector (excitation 268 nm, emission 440 nm). The mobile phase was dichloromethane-acetonitrile (85:15) with a flow rate of 1 ml min^{-1} . Calculations were performed with an external lolitrein B standard solution (supplied by R. C. Gallagher, AgResearch, New Zealand). The determined limit of quantification was 0.18 $\mu\text{g g}^{-1}$ of dry plant material.

Ergovaline was analysed according to an adaptation of the method of Hill *et al.* (1993). Samples were extracted by alkaline chloroform with an internal standard (ergotamine 500 ng ml^{-1} in chloroform) and shaken for 2 h at room temperature. Extracts were filtered and purified on a silica column (Ergosil Analtech Newark, DE, USA) prewashed with chloroform. Pigments were removed by washing with chloroform-acetone (1:3). Ergot alkaloids were eluted with methanol and the eluent was evaporated under a nitrogen stream at 30°C . The dry residue was dissolved in methanol. The HPLC analysis was performed on a Thermoquest liquid chromatograph system equipped with a Si C-18 (Zorbax XDB) column (150 mm \times 4.6 mm, 20 μl injection) and a fluorescence detector (excitation 250 nm, emission 420 nm). The mobile phase was acetonitrile (36, 5%) in aqueous ammonium carbonate (200 mg l^{-1}) run at 1.1 ml min^{-1} .

A standard was prepared by adding ergovaline (from F. Smith, Department of Pharmacal Sciences, Auburn University, AL, USA) and ergotamine (Sigma Chemical) to 500 mg

of a noninfected ryegrass sample up to 500 ng g^{-1} and treated as described above. The limit of quantification of the method was 0.15 $\mu\text{g g}^{-1}$ of dry plant material.

Peramine was analysed by a method supplied by B.A. Tapper (AgResearch, New Zealand). Dried ground straw was mixed with 40% propan-2-ol for 30 min at 90°C . After centrifugation, the crude extract was cleaned-up on a Bond Elut (BA column) (Varian) conditioned with 80% aqueous methanol containing 2% ammonium hydroxide and then with pure methanol. Peramine was eluted from the column with a 5% formic acid - 80% methanol solution. The eluent was dried under a nitrogen stream and the residue resuspended in methanol. The HPLC analysis was performed using a Thermoquest liquid chromatograph system equipped with a silica column (250 mm \times 4.6 mm, 20 μl injection) and an UV detector (280 nm). The mobile phase was a buffer consisting of 50 mM ammonium acetate, 5 mM guanidinium carbonate and 0.2% acetic acid in 18% aqueous methanol at a flow rate of 1 ml min^{-1} . An endophyte-free ryegrass sample supplemented with peramine and extracted as described above was used as external standard for calculation. The limit of quantification of this method was 2 $\mu\text{g g}^{-1}$ of dried plant material.

Statistical procedures

Linear growth data were subjected to variance analysis using the General Linear Model (GLM) procedure of SAS. In a first analysis, the main effect was the endophyte strains and the means were grouped by the cluster method developed by Scott & Knott (1974). The advantage of this method is to partitionate the mean values into nonoverlapping groups. In a second variance analysis, the morphological groups were designated as the main effect and the means were separated by the test of Bonferroni, which is adapted to unequal cell-size.

The production of lolitrein B for two distinct morphological groups of endophytes was compared by a two-sample *t*-test (procedure TTEST in SAS). The distribution obtained for the peramine was not Gaussian, leading to compare the medians by a nonparametrical test (proc. NPARIWAY in SAS).

Results

Success of isolation

Isolates were obtained in all cases except one. The delay in the isolate emergence was extremely variable, from 7 to 33 d (median value, 14 d) but generally consistent within seed lots.

In 70 plants, the colonies growing from different seeds of the same lot were homogenous in their morphology and growth rate and were considered to belong to one isolate. However, for 12 plants, two different morphologies could be

distinguished among the colonies from the same seed lot. These differences in morphology remained stable when the colonies were subcultured. The two variants were considered as different isolates, distinguished by the letters A and B in Table 1. Thus, the total number of isolates studied was $70 + (12 \times 2) = 94$.

Species identification

Species identification was carried out by the isozyme patterns of MDH1, PGI and PGIM. All the isolates were identified by this method: 83 were found to belong to *Neotyphodium lolii*, 7 to LpTG-2 and 4 to *Gliocladium*-like (Table 1). Seeds from 12 plants harboured two different isolates. In one case, an isolate of *Gliocladium*-like was obtained as well as with an isolate of *N. lolii*. In three cases, isolates of LpTG-2 and *N. lolii* were obtained. In eight cases, two different isolates of *N. lolii* were found in the same seed lot.

Morphology of cultures on PDA

The isolates were assigned to eight stable and well-characterized Morphological Groups (MG) on PDA (Fig. 2).

Morphological Group I (MG I, Fig. 2a) consisted of slow-growing isolates which develop vertically more than horizontally. The mycelium is strongly aggregated. Vertical development is accompanied by the formation of irregular crests and convolutions. This morphology is often described as 'brain-like'. The margin of the colony is vertical. The colony is waxy, without aerial mycelium.

In MG II, the centre of the colony resembles MG I, but the margin is made of horizontal, smooth mycelium (Fig. 2b). Aerial mycelium is absent or scarce.

MG III is the most heterogeneous; it includes two subtypes which both are originally flat and smooth. In subtype A, domes or warts made of aerial, white mycelium erupt at the surface after 10–15 d of growth (Fig. 2c), they can later fill the centre of the colony or constitute sectors. In subtype B, the centre of the colony is white and slightly raised; after 15 d, it appears scored by furrows which are both radial and concentric. These two morphologies have been included in one group because some isolates alternately showed one or the other morphology according to the subcultures.

MG IV is characterized by a high radial growth rate. The colony is flat and smooth, with very few or no aerial mycelium (some tufts in the central part). The margin, made of intramatricial mycelium, appears diffuse (Fig. 2d).

In MG V, as in the previous type, the colony is flat, with a diffuse margin. After 15 d, it is covered in its major part with aerial, powdery or granular mycelium (Fig. 2e). This group resembles group VIII (*Gliocladium*-like), however, the lower side is rimmed with light grey, nor with olive green.

The colony characterizing MG VI is flat, smooth, waxy, almost mucose, without aerial mycelium or white warts. The

centre of the colony is typically grooved by curved, radial furrows (Fig. 2f).

The colony characterizing MG VII has an irregular outline. Its surface is cottony, and entirely covered with aerial mycelium. The margin is diffuse, consisting of superficial hyphae (Fig. 2g).

In the last group, MG VIII, the colony is circular, white, flat, and covered with powdery or granular aerial mycelium (Fig. 2h). The agar on the under side is tinted with olive green (permitting a clear distinction from group V).

The allocation of the 94 isolates obtained to Morphological Groups is shown in Table 1.

The seven isolates belonging to species LpTG-2 all belonged to MG VII and were the only isolates in this group. The four isolates identified as *Gliocladium*-like were the only ones in MG. The isolates identified as *Neotyphodium lolii* were allocated between MG I to VI, with the following distribution: MG I: five isolates; MG II: 26; MG III: 39; MG IV: five; MG V: four; MG VI: four. The six reference isolates obtained from AgResearch Grassland (R1–R6) had the following distribution: R1 and R2 (LpTG-2); MG VII: R3, R4, R5 and R6 (*N. lolii*); MG VI, V, V and I, respectively.

Growth rate

Analysis of the linear growth rate of the isolates led to the definition of nine nonoverlapping groups as shown in the last column of Table 1.

Figure 3 shows the distribution of linear growth for the eight Morphological Groups (MG). The main effect of MG on growth rate was highly significant ($P = 0.001$). According to the comparison of means, the different MG are classified in four groups of growth. MG IV and MG V belong to the group showing the significantly highest growth rate. LpTG-2 (MG VII) and *Gliocladium*-like (MG VIII) belong to a second group which overlaps the set including MG IV and MG V. MG II and MG III, which contain the majority of isolates, are classified in a third, distinct group of intermediate growth. MG I and MG VI constitute a fourth group characterized by significantly lower growth rates.

Mycotoxins

The analyses were carried out for a total of 79 plants representing 79 populations. For lolitrem B, the results of the 12 plants from which two different fungal isolates had been isolated were considered separately. Among the 67 plants which harboured only one isolate 11 did not contain lolitrem B and 56 contained lolitrem B at concentrations higher than the quantification threshold ($0.18 \mu\text{g g}^{-1}$). Of the 11 plants without lolitrem B, three plants harboured a *Gliocladium*-like fungus; that is no plant harbouring only a *Gliocladium*-like isolate synthesized lolitrem B. A further four plants harboured an isolate of LpTG-2. That is, no plant harbouring only an isolate

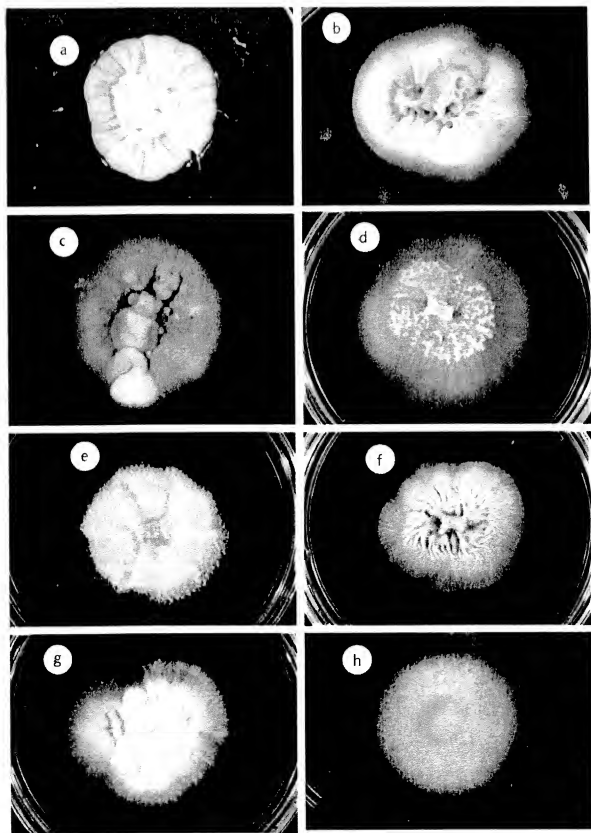


Fig. 2 Macromorphology of the eight Morphological Groups (MG) on PDA: a–f: *Neotyphodium folii*, g: LpTG-2, h: *Gliocladium*-like.

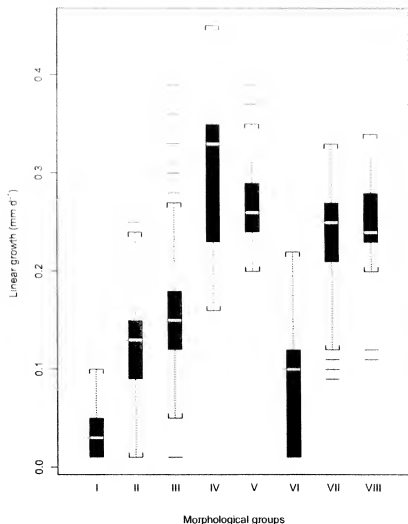


Figure 3 Relationship between increase in colony diameter (mm d^{-1}) and morphological groups. The horizontal, white line in each box is the median of the data; the height of the box is equal to the interquartile distance (IQD). The vertical line corresponds to a distance $1.5 \times \text{IQD}$ from the median. Data points falling outside this interval are indicated by horizontal, black lines

of LpTG-2 synthesized lolitrem B. Another four plants harboured an isolate of *Neotrophodium lolii* belonging to MG I. That is, no plant with an isolate in MG I synthesized lolitrem B.

The 56 plants containing lolitrem B did so at concentrations ranging from 0.8 to $5.75 \mu\text{g g}^{-1}$ (Fig. 4a) with an average value $2.90 \mu\text{g g}^{-1}$. All these isolates belonged to *N. lolii* and were distributed between MG II (22 isolates), MG III (31 isolates) and, marginally, MG IV (1) and MG V (2). MG IV and V were very small and the mean values for lolitrem B concentrations were compared only between MGs II and III, with MG II isolates producing significantly more lolitrem B ($P = 0.0063$).

In nine of the 12 plants from which two different isolates had been obtained, both isolates belonged to *N. lolii*. In these plants, lolitrem B was present at concentrations ranging from 0.80 to $4.30 \mu\text{g g}^{-1}$. In three plants where there was one isolate of *N. lolii* and one isolate of LpTG-2 no lolitrem was detected in two cases, and one plant contained $2.1 \mu\text{g g}^{-1}$ lolitrem.

Among the 67 plants that harboured only one isolate, 41 contained ergovaline, at concentrations higher than the

quantification threshold of $0.15 \mu\text{g g}^{-1}$. The plants without ergovaline included three plants harbouring *Gliocladium*-like isolates and 23 plants harbouring *N. lolii*. Among these 23 isolates, 22 belonged to MG II and only one to MG IV. Correspondingly, ergovaline was never detected in plants in which only one isolate of MG II had been found.

The 41 plants containing ergovaline, did so at concentrations ranging from 0.24 to $3.46 \mu\text{g g}^{-1}$ with an average value of $1.14 \mu\text{g g}^{-1}$. Among these, four belonged to LpTG-2 and 37 to *N. lolii*, and were distributed between MG I (four isolates), MG III (31 isolates) and MG V (two isolates). Interestingly, the highest concentrations were observed in two plants harbouring a MG I isolate.

For the plants containing two different isolates, in three cases a LpTG-2 isolate was associated with an isolate of *N. lolii* of MG III or MG IV, and these plants contained ergovaline. Four associations involved two isolates of *N. lolii* belonging to MG groups other than MG II and ergovaline was also present. In four situations in which MG II was involved in association

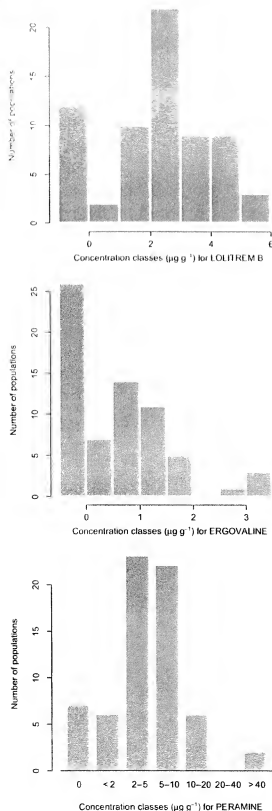


Fig. 4 Frequency distribution of mycotoxin concentrations. (a) lolitrem B; (b) ergovaline; (c) peramine.

with isolates of other groups, ergovaline was absent in three cases and present in one case. The last association was between *Gliocladium*-like and an isolate of *N. lolii* MG III and the plant contained ergovaline.

The quantification threshold was higher for peramine than for the other two mycotoxins ($2 \mu\text{g g}^{-1}$). Thus, in some cases peramine was detected, but could not be quantified. Among the 66 plants which harboured only one isolate, seven had no detectable peramine, six showed only traces of the mycotoxin (concentrations $< 2 \mu\text{g g}^{-1}$) and 53 contained higher concentrations. The absence of peramine was not linked to absence or presence of ergovaline or lolitrem and could not be related to a special morphology of the isolate in culture. 53 plants contained peramine at concentrations ranging from 2.0 to $52.8 \mu\text{g g}^{-1}$, according to an asymmetrical distribution, with a median value $6.30 \mu\text{g g}^{-1}$, (Fig. 4c). Two plants showed a particularly high concentration of peramine (52.8 and $41.3 \mu\text{g g}^{-1}$). Peramine production was significantly higher for the isolates of MG III than for those of MG II.

For those plants from which two different isolates had been obtained, the peramine concentrations showed no consistent pattern.

Discussion

Isolation of endophytes from germinating seeds was very successful. The 94 isolates obtained belonged to the three endophyte species which had already been described in *Lolium perenne*: *Neotyphodium lolii*, LpTG-2 and the 'Gliocladium-like' fungus. *N. lolii* was the most common, representing 90% of the isolates. No other species were detected. The specific identification of these three species was based on their isozyme patterns for the two enzymes MDH and/or PGM, as described by Christensen *et al.* (1993) and Naffaa *et al.* (1998). The presence of conidia in culture for LpTG-2 at 20–24°C (*N. lolii* is always sterile at this temperature), is another criterion advocated to distinguish between these two clavicipitaceous species (Christensen *et al.*, 1993). The nine isolates of LpTG-2 isolated in our study were checked for the presence of conidia on PDA at 23°C. Conidia were observed for four isolates only. On the other hand, they were not observed on the cultures of five isolates of *N. lolii* which had been drawn by lot. The presence of conidia in cultures of LpTG-2 at room temperature seems to be a sufficient, but not necessary, condition for identification.

Pure culture macromorphology may be useful for species identification, since LpTG-2 and *Gliocladium*-like show a specific and very homogenous morphology (MG VII and MG VIII, respectively). *Neotyphodium lolii* varies greatly in morphology and in growth rate. Linear growth was preferred to biomass production as being potentially more discriminant. The strong correlation observed between macromorphology and linear growth is not surprising: the very slow apical growth of the mycelial hyphae of some strains is concomitant with a

high rate of branching, the result being a three-dimensional growth of the colony, which is stroma-like, being as high as wide. This morphology, which has sometimes been named 'brain-like', characterizes MGs I and II, in MG II, the central pseudohyphae is surrounded by a band of flat mycelium while in MG I, it falls abruptly into the culture medium. These two groups are clearly distinct from each other and from all the other morphological types which can be described within *N. lolii*. The morphologies I and II appear stable with subculturing and are retained after a storage at low temperatures (-80°C). The flat aspect of the young colony characterized all the other morphological groups described: the MGs IV, V and VI show typical morphological aspects, and contain only a few isolates, MG III is less homogeneous; in this group, the centre of the colony can undergo different development after 20 d of growth: for example appearance of cottony warts or of radial and concentric furrows. This type is the one that contained the highest number of isolates. The typical appearance of the colonies of LpTG-2 on PDA has already been described by Christensen *et al.* (1991). These authors had also reported the high variability of the isolates of *N. lolii* and published photos resembling our groups II and III.

The variability within *N. lolii* was also considerable with respect to the synthesis of the three main mycotoxins: lolitrem B, ergovaline and peramine. Four isolates, all of French origin (6% of the isolates) did not synthesize lolitrem B. 23 isolates (37%) did not synthesize ergovaline. Of these 23, 20 originated from France, one from Poland, one from Yugoslavia and one from Greece. Six isolates (10%) (excluding those with traces of peramine lower than the quantification threshold) did not synthesize peramine. Among these six, all of French origin, one was also lolitrem B-deficient, two were ergovaline-deficient, and three synthesized both ergovaline and lolitrem B.

The most striking observation was the close link between the deficiency for ergovaline or lolitrem B in *N. lolii* and the morphology of the isolates: all four isolates which did not produce lolitrem B were from MG I and no isolate of MG I produced lolitrem B. Among the 23 isolates that did not produce ergovaline, 22 belonged to MG II. In MG II, no isolate produced ergovaline. By contrast, the peramine-deficient strains distributed among several morphological groups, including MG I and MG III.

As the MG I and MG II morphologies coincide with a slow linear growth rate, it seems that the 'mutation' (in the broad sense) which led to the absence of synthesis of lolitrem or ergovaline is regularly accompanied by disturbances of the mycelial growth. The loss of the ability to synthesize ergovaline (or lolitrem) could be the result of a chromosome deletion which could also result in the loss of genes playing a role in mycelial growth and morphogenesis. As the deficiencies for ergovaline and lolitrem are independent and accompanied by different morphological disturbances, the two deletions probably concern different chromosomes or chromosome fragments. The loss of a minor chromosome (a 'B chromosome',

according to Kistler & Mra (1992)) could also be an explanation: Kuldau *et al.* (1999) found eight chromosomes in *N. lolii* of which three were smaller than 3 Mb.

Figure 4 shows the frequency distribution of the concentrations of the three mycotoxins in the plants. The concentrations of lolitrem B and ergovaline are similar to those in the literature (Di Menna *et al.*, 1992; Ball *et al.*, 1997b; Lane *et al.*, 1997a, 1997b). By contrast, the concentrations of peramine (median value: $6.3 \mu\text{g g}^{-1}$ for 55 isolates) are lower than those in the literature, which are often in the range of $10\text{--}30 \mu\text{g g}^{-1}$ (Ball *et al.*, 1995a, 1995b, 1997a). However, for two particular populations, the concentrations were much higher ($> 40 \mu\text{g g}^{-1}$). The genotype of the fungus, which controls the synthesis of a given mycotoxin, can also play a role in fixing the quantitative level of this mycotoxin within the plant. However, many other factors probably play a role, such as the host genotype, the mycelium density in the host tissues and various environmental factors; so it is difficult to assert that differences recorded in the three mycotoxins concentrations faithfully reflect genetic quantitative differences between the fungal isolates.

The isolates of MG II (which do not produce ergovaline) produced significantly more lolitrem B than the isolates of MG III. The isolates of MG III produce significantly more peramine than those of MG II. However, there was no correlation between the concentration of the three mycotoxins if all isolates are considered together.

Isolates deficient in both ergovaline and lolitrem B were not found. The predicted frequency of such strains would be the product of the individual frequencies: $6\% \times 37\% = c. 2\%$. This low predicted frequency could explain why no such isolate was found. On the other hand, if it is true that both the ergovaline-free and the lolitrem B-free isolates of *N. lolii* are the result of genetic accidents (for instance of chromosome deletions), one would expect that the isolates carrying a double deletion would be particularly deficient and find it difficult to survive in the plant. However, screening of more than 1000 populations in New Zealand, has succeeded in finding such isolates (Fletcher & Easton, 1997).

The close link between the absence of ergovaline or lolitrem B and a specific morphology of the colonies in pure culture could, if it is confirmed on a higher number of strains from more various origins, considerably improve the process of selection of harmless strains. Such a selection could be carried out by laboratories equipped only for routine mycology and lacking the complex equipment and technical competence necessary for the analysis of mycotoxins by HPLC.

This study confirmed that the species LpTG-2, of hybrid origin (Schardl *et al.*, 1994), is in a minority among the endophytes of *L. perenne* in France. Only three French isolates of this taxon were found in our study: two from the region of Marseille and one from Corsica. Four other isolates were isolated from populations from Italy and Spain. The two reference isolates obtained from AgResearch also have a

Western Mediterranean origin. Thus, LpTG-2 appears to be a West Mediterranean species.

The seven LpTG-2 isolates were homogeneous in their morphology, growth rate, isozyme pattern and mycotoxin synthesis (lohitrem B is not synthesized). The isozyme patterns MDH and PGM allow a certain identification of the species, but the morphology of the colonies on PDA may appear to be a safe method of identification.

The taxon '*Glucadium-like*' was also a minority among our isolates. The four isolates obtained appeared identical as concerned their isozyme pattern, macro-morphology (MG VIII type), high growth rate, low isolation delay, and preference for cool temperatures (18°C). The populations concerned came from Northern France, Central France and Germany. As for LpTG-2, the identification can be easily carried out from the macro-morphology alone. Neither conidiophores nor conidia were observed in *planta* or in culture.

In 12 cases (14%) two different endophytes were isolated from the same seed lot. As each lot had been harvested from only one plant, two different fungal isolates must have coexisted within the same plant. In one case, a *Glucadium-like* fungus was found associated with an isolate of *N. lolii*. In three cases, the association was between LpTG-2 and *N. lolii* and in eight cases, between two different isolates of *N. lolii*. Indeed, we could only detect the situations in which the two associated isolates of *N. lolii* belonged to two different MGs. It is probable that other situations exist in which the two (or more) isolates are morphologically similar and could be detected only with molecular markers.

Coexistence between two endophytes in the same plant has already been reported for the associations between a clavicipitaceous and a nonclavicipitaceous endophyte (Schmidt, 1994; Siegel *et al.*, 1995). The association between two different isolates of the same species of endophyte has already been reported and analysed by Meijer & Leuchtmann (1999) on the pair *Brachypodium sylvaticum*/*Epichloë sylvatica*. However, to our knowledge, the present work is the first mention of the presence of two different strains of *Neotyphodium lolii* in the same plant of *Lolium perenne*. Frequent coexistence between two clavicipitaceous endophytes in the same host may support parasitic hybridization in the continuum existing from parasitic *Epichloë* to mutualistic *Neotyphodium*, leading to new species.

Acknowledgements

The work was carried out with the technical collaboration of C. Astier, P. Desray, C. Huc and M. Carcelen. The authors gratefully acknowledge Mrs. Maureen Bocquet for her reading of the English manuscript.

References

- Aasen AJ, Culveron CCJ, Finnie EP, Kellogg AW, Smith LW. 1969. Alkaloids as a possible cause of ryegrass staggers in grazing livestock. *Australian Journal of Agricultural Research* 20: 71–86.

- Ballaourier I, Charmet G. 1991. Relationships between agronomic characters and ecogeographical factors in a collection of French perennial ryegrass populations. *Agronomie* 11: 645–657.
- Ballaourier I, Imbert C, Charmet G. 2000. Evidence for phylogeographic structure in *Lolium* species related to the spread of agriculture in Europe. *Journal of Applied Ecology* 101: 131–138.
- Ball OJP, Barker GM, Prestidge RA, Laurence JR. 1997a. Distribution and accumulation of the alkaloid peramine in *Neotyphodium lolii*-infected perennial ryegrass. *Journal of Chemical Ecology* 5: 1419–1434.
- Ball OJP, Barker GM, Prestidge RA, Sprosen JM. 1997b. Distribution and accumulation of the mycotoxin lolitrem B in *Neotyphodium lolii*-infected perennial ryegrass. *Journal of Chemical Ecology* 5: 1435–1449.
- Ball OJP, Lane GA, Prestidge RA. 1995a. *Acetaminium lolii*, ergovaline and peramine production in endophyte-infected perennial ryegrass. *Proceedings of the 48th New Zealand Plant Protection Conference*: 224–228.
- Ball OJP, Prestidge RA, Sprosen JM. 1995b. Interrelationships between *Acetaminium lolii*, Peramine, and Lolitrem B in Perennial Ryegrass. *Applied and Environmental Microbiology* 61: 1527–1533.
- Charmet G, Ballaourier I, Bion A. 1990. Agronomic evaluation of a collection of French perennial ryegrass populations: multivariate classification using genotype-environment interactions. *Agronomie* 10: 807–823.
- Charmet G, Ballaourier I, Ravel C. 1993. Isozyme polymorphism and geographic differentiation in a collection of French perennial ryegrass populations. *Genetic Resources and Crop Evolution* 40: 77–89.
- Christensen MJ, Latch GCM, Tapper BA. 1991. Variation within isolates of *Acetaminium endophytes* from perennial ryegrass. *Mycological Research* 88: 918–925.
- Christensen MJ, Leuchtmann A, Rowan DD, Tapper BA. 1993. Taxonomy of *Acetaminium* endophytes of tall fescue (*Festuca arundinacea*), meadow fescue (*F. pratensis*) and perennial ryegrass (*Lolium perenne*). *Mycological Research* 9: 1083–1092.
- Cunningham IJ, Hartley WJ. 1959. Ryegrass staggers. *New Zealand Veterinary Journal* 7: 1–7.
- Di Menna ME, Mortimer PH, Prestidge RA, Hawkes AD, Sprosen JM. 1992. Lolitrem B concentrations, counts of *Acetaminium lolii* hyphae, and the incidence of ryegrass staggers in lambs on plots of *A. lolii*-infected perennial ryegrass. *New Zealand Journal of Agricultural Research* 35: 211–217.
- Fletcher LR, Easton HS. 1997. The evaluation and use of endophytes for pasture improvement. In: Bacon CW, Hill NS, eds. *Third International Symposium on Neotyphodium/Grass Interactions*. Athens, GA, USA: Plenum Press. 209–225.
- Fletcher LR, Harvey IC. 1981. Association of a *Lolium* endophyte with ryegrass staggers. *New Zealand Veterinary Journal* 29: 185–186.
- Gallagher RT, Hawkes AD, Stewart JM. 1985. Rapid determination of the neurotoxin lolitrem B in perennial ryegrass by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography* 321: 217–226.
- Glenn AE, Bacon CW, Price R, Hanlin RT. 1996. Molecular phylogeny of *Acetaminium* and its taxonomic implications. *Mycologia* 5: 369–383.
- Guérin P. 1898. Sur la présence d'un champignon dans l'ivraie. *Journal de Botanique* 12: 230–238.
- Hill NS, Rottinghaus GE, Agee CS, Schultz LM. 1993. Simplified sample preparation for HPLC analysis of ergovaline in tall fescue. *Crop Science* 33: 331–333.
- Kisler HC, Miao VP. 1992. New modes of genetic changes in filamentous fungi. *Annual Review of Phytopathology* 30: 131–152.
- Koldau GA, Tsai HF, Schardl CL. 1999. Genome sizes of *Epichloë* species and anamorphic hybrids. *Mycologia* 5: 776–782.
- Lane GA, Tapper BA, Davies E, Hume DE, Latch GCM, Barker DJ, Easton HS, Rolston MP. 1997a. Effect of growth conditions on alkaloid concentrations in perennial ryegrass naturally infected with endophyte. In: Bacon CW, Hill NS, eds. *Third International Symposium on Neotyphodium/Grass Interactions*. Athens, GA, USA: Plenum Press. 179–182.

- Lane GA, Lapper BA, Davies I, Davidson C. 1997b. Epizalium distribution on perennial ryegrass naturally infected with endophyte. In: Bacon CW, Hill NS, ed. *Third International Symposium on Nectrophidium/Grass interactions*. Adelaide, CA, USA: Plenum Press, 65–67.
- Latch GM. 1989. Plant improvement using endophyte fungi. In: *Association Française pour la Production Fourragère*, ed. *VII International Grassland Congress*. Poissy, France: Association Française pour la Production Fourragère, 345–346.
- Latch GM. 1999. Influence of *Acremonium* endophytes on perennial ryegrass improvement. *New Zealand Journal of Agriculture Research* 32: 511–518.
- Latch GCM, Christensen MJ, Samuels GJ. 1984. Five endophytes of *Lolium* and *Festuca* in New Zealand. *Myrmason* 20: 535–550.
- Lewis GC, Ravel C, Nalaa W, Astier C, Charnet G. 1997. *Acremonium* endophytes in wild populations of *Lolium* spp. in European countries and a relationship between level of infection and climate in France. *Annals of Applied Biology* 130: 227–238.
- McClelland I. 1920. The endophytic fungus of *Lolium*. Part I. *Proceedings of the Royal Society of Victoria* 11: 252–301.
- Mejer G, Leuchmann A. 1999. Multistress infections of the grass *Brachiopodium sylvaticum* by its fungal endophyte *Epibolia sylvatica*. *New Phytologist* 141: 355–368.
- Morgan Jones G, Gams W. 1982. Notes on Hyphomycetes. XII. An endophyte of *Festuca arundinacea* and the anamorph of *Epibolia typhina*, new taxa in one of two new sections of *Acremonium*. *Myrmason* 15: 511–518.
- Nalaa W, Ravel C, Guillaumin JJ. 1998. A new group of endophytes in European grasses. *Annals of Applied Biology* 132: 211–226.
- Philpott MN. 1989. A symptomless endophyte of ryegrass (*Lolium perenne*) that spores on its hosts – a light microscope study. *New Zealand Journal of Botany* 27: 513–519.
- Ravel C. 1997. *Les champignons endophytes de graminées: distribution et symbiose avec leur hôte*. PhD thesis, Université Blaise Pascal, Clermont-Ferrand, France.
- Sampson K. 1933. The systemic infection of grasses by *Epibolia typhina* (Pers.) Tul. *Transactions of the British Mycological Society* 18: 30–40.
- Sampson K. 1937. Further observations on the systemic infection of *Lolium*. *Transactions of the British Mycological Society* 21: 84–97.
- Schardl CL, Leuchmann A, Tsai TH, Gallett MA, Watt DM, Scott DB. 1999. Origin of a Fungal Symbiont of Perennial Ryegrass by Interspecific Hybridization of a Mutualist with the Ryegrass Choke Pathogen, *Epibolia typhina*. *Genetics* 156: 1307–1317.
- Schmidt D. 1994. Du nouveau sur les endophytes de la legume des prés. *Revue Suisse d'Agriculture* 1: 45–65.
- Scott AJ, Knott M. 1974. A cluster analysis method for grouping means in the analysis of variance. *Biometrics* 30: 507–512.
- Siegel MR, Schardl CL, Philips TD. 1995. Incidence and compatibility of non-Clavicipitaceous fungal endophytes in *Festuca* and *Lolium* grass species. *Myrmason* 2: 196–202.

FUNGAL ENDOPHYTES OF GRASSES

M. R. Siegel

Department of Plant Pathology, University of Kentucky, Lexington, Kentucky 40546-0091

G. C. M. Latch

Department of Scientific and Industrial Research, Palmerston North, New Zealand

M. C. Johnson

Department of Biological Sciences, Georgetown College, Georgetown, Kentucky 40324

INTRODUCTION

The term endophyte (Greek: *endo* = within + *phyte* = plant) has been defined as an organism contained or growing (entirely) within the substrate plant, whether parasitically or not (103, 106). Using this term in its broadest sense, the subject of this chapter could include all fungi that spend all or nearly all of their life cycles in the host grasses. We do not discuss such endophytic fungi as the smuts and vesicular-arbuscular mycorrhizas that infect grass. Instead, our interests lie primarily with a specific group of clavicipitaceous fungi that belong or are related to fungi in the tribe Balansiae (5, 24). These fungi either are true endophytes that never produce external fruitifications on the plant or else may produce external mycelium and/or spores that affect flower and seed production. We focus specifically on those grass-endophyte complexes that may also cause maladies of grazing animals.

Using the above criteria, two recent events involving grass-endophyte associations have important implications for the livestock industry. In 1977 Bacon et al (9) reported the close association of an endophyte (*Sphacelia typhina*) in infected tall fescue (*Festuca arundinacea*) and the incidence of

293

0006-4286/87/0901-0293\$02.00

fescue toxicosis (summer syndrome) in cattle. A similar association between a then unidentified endophyte in perennial ryegrass (*Lolium perenne*) and the incidence of ryegrass staggers in sheep in New Zealand was reported in 1981 by Fletcher & Harvey (27).

Since the initial work of Bacon et al (9) in 1977, researchers have come to further understand the relationship between fungal endophytes of grasses and animal toxicoses. This understanding includes the origin and incidence of infected grasses, modes of dissemination of the fungi, identification of the chemicals responsible for toxicoses, and control of the fungi. Specifically, what has been learned is that endophyte-infected tall fescue and perennial ryegrass are widely distributed in the United States and New Zealand, respectively; endophytic fungi are only seed disseminated; specific chemicals are responsible for fescue toxicosis and ryegrass staggers; infected pasture grasses cost the livestock producer hundreds of millions of US dollars annually in lost production; the fungi cannot be controlled in the field, but endophyte-free cultivars do result in greatly improved livestock production in the United States; and lastly, the relationship between grass and symbiont is primarily mutualistic, resulting in many benefits to host and fungus (5, 10, 97). With regard to this final point, it is now recognized that these grass-endophytes can play an important role in survival of the host plants subjected to environmental stresses (insects, grazing animals, drought, and heat). The potential for improved insect resistance of endophyte-infected grasses has already been exploited by the selection of naturally infected cultivars of *Festuca* and *Lolium* species (29, 30).

This chapter, therefore, reviews the harmful and beneficial effects of endophytic fungi on forage and turf grasses and on grazing animals. It includes a historical perspective on the fungi (incidence, dissemination, and control), the relationship between infected grasses and animal toxicoses, the nature of the grass-fungus interaction, and the value of infected and noninfected cultivars in pasture and turf.

HISTORICAL PERSPECTIVES

The presence of endophyte mycelium in the seed of a grass (*Lolium temulentum*) was first recorded by Vogl in 1898 (105), but the earliest known specimen of this host and fungus dates back 4,400 years to seeds found in the tomb of a Fifth Dynasty Egyptian Pharaoh (64). Vogl's (105) discovery aroused interest because seeds of *L. temulentum* were known to be toxic to animals, and there was speculation that the fungus was responsible. Other species of *Lolium* were also found to contain mycelium in a high proportion of their seeds; thus, the endophytic nature of the fungus in plants was established (28, 76).

ilar association between a *Lolium perenne*) and the and was reported in 1981

researchers have come to endophytes of grasses and origin and incidence of the fungi. Specifically, tall fescue and perennial and New Zealand, respectively, specific chemicals are used on pastures worth \$5 dollars annually in lost field, but endophyte-free production in the United States is primarily fungus (5, 10, 97). With these grass endophytes can be subjected to environmental heat. The potential for grasses has already been cultivated of *Festuca* and

and beneficial effects of on grazing animals. It is, hence, dissemination, and and animal toxicoses, the role of infected and non-

red of a grass (*Lolium* 5), but the earliest known ears to seeds found in the Vogl's (105) discovery were known to be toxic to was responsible. Other in a high proportion of in plants was established

Classification

Endophytes of grasses and sedges are grouped in the tribe Balansiae of the Clavicipitaceae. Within this tribe are the genera *Balansiosipsis*, *Atkinsonella*, *Myriogenospora*, *Balansia*, and *Epichloe* (24, 66). The taxonomy of endophytes, for which no teleomorphic state has been found, is still being debated. Morgan-Jones & Gams (70) created the section *Albo-lanosa* in the genus *Acremonium* to accommodate the endophyte commonly found in tall fescue. They named this fungus *A. coronophialum*, and subsequently, endophytes in other species of *Festuca* and *Lolium* have been placed in this section (59, 116, 117). These endophytes may be related to the clavicipitaceous teleomorph *Epichloe* (59, 70), but they have temporarily or permanently lost the ability to form sexual spores. Rykard et al (91) argue that it would be preferable to leave these newly named *Acremonium* species in the genus *Sphacelia*, the conidial state of *Epichloe typhina*. This question will only be resolved when the teleomorphic states of the fungi are found.

Latch et al (59) have described two seed-borne endophytes in perennial ryegrass and tall fescue with *Gliocladium*-like and *Phialophora*-like conidia, respectively. The relationship of these endophytes to those in the Balansiae is unclear.

All these Balansiae, except for *Myriogenospora*, produce systemic infections in leaves, culms, and inflorescences, but most become conspicuous only when sporulation occurs as the plant flowers. Many endophytes prevent their host from flowering, whereas others do not interfere with the plant's reproduction, and indeed, their mycelium may be present in the seed. Inter-cellular hyphae have been found in the ovary wall and throughout the nucellar tissue of *F. versuta*, tall fescue, and perennial ryegrass ovules (18, 65, 78). However, in some perennial ryegrass megagametophytes, hyphae of *Acremonium lolii* (59, 61) become intracellular when they breach the embryo sac and enter the antipodal cells (78). As the embryo enlarges and differentiates, the hyphae pass into the shoot apical region. Thus, some endophytes are very efficiently dispersed with their host seed and apparently have no need for spores as a means of dissemination. Indeed, it is believed that the only means of dissemination of *Acremonium* endophytes is through maternal transmission in infected seed (4, 54, 96).

Incidence

Species of *Balansia*, *Balansiosipsis*, *Atkinsonella*, and *Myriogenospora* infect a wide range of grasses commonly found in the tropics that have a C₄-type photosynthetic pathway. Most of these grasses are regarded as weeds or as having minor agricultural importance (24, 66). *Epichloe typhina* is found on important genera of forage and turf grasses such as *Agrostis*, *Dactylis*, *Festuca*, *Holcus*, *Hordeum*, *Lolium*, and several other genera of minor agri-

cultural importance. The striking choke symptom of mycelium around the inflorescence is present on most infected plants (24, 92).

Endophyte, that have *Acremonium*, *Sphaeria*, *Gliocladium*-like, or *Phialophora*-like anamorphic states and that produce no external fructifications on grasses were originally found only in species of *Festuca* and *Lolium* (9, 59, 70). Recent searches have extended the host range to include species of *Bromus*, *Poa*, and *Stipa* (111, 115, 117). Undoubtedly, further searches will extend this host range.

The incidence of endophytes in tall fescue, perennial ryegrass, and Italian ryegrass (*Lolium multiflorum*) has been studied because these grasses are of prime importance in many countries. Tall fescue is grown on 12-14 million hectares in the United States, and the two ryegrasses are the main pasture grasses sown in Europe and New Zealand. Endophytes have been found in seeds of these three grasses collected from areas of Europe where the species are indigenous and may possibly have originated (61, 96). The seed-borne nature of endophytes has resulted in their being spread, along with their hosts, throughout the world. Over 90% of tall fescue pastures in the United States contain plants infected with *Acremonium coenophialum* (97, 108). A survey by the Auburn University fescue diagnostic laboratory of samples from twenty-six states showed that 94% of pastures contained endophyte, and the plants in these pastures had a mean infection of 58% (95). The dominant cultivar of tall fescue in the United States is Kentucky-31, and most plants are infected with endophyte (96); however, new cultivars with a low incidence of endophyte have now been developed (97).

Seed samples from 53 of 64 old pastures of perennial ryegrass from eight European countries were found to be infected with the endophyte *A. lolii* (61). Recently sown pastures probably have a much lower incidence of endophyte because a survey of sixteen current European cultivars showed that only four contained endophyte, the average incidence being 15% in these cultivars (61). In New Zealand, the majority of plants in seven million hectares of perennial ryegrass are infected with *A. lolii*, and the two most commonly sown cultivars have 65-95% of the plants infected (54, 94, 97). There is little information on the incidence of an *Acremonium*-like endophyte in Italian ryegrass pastures. In Europe it appears to be low, especially among recent cultivars, but in New Zealand over 50% of plants of new Italian ryegrass cultivars may be infected with this endophyte (57, 61).

Methods of Detection

Grasses infected with endophytes that have as their anamorphic state *Acremonium*, *Gliocladium*-like, or *Phialophora*-like conidia are symptomless. The mycelium is intercellular within the host plants, but several techniques are available for detection and identification of these endophytes.

mycelium around the seed.

Gloecladium-like or no external fruiting bodies have been found in Europe where the species *Festuca* and *Lolium* range to include species studied; further searches

nal ryegrass, and Italian use these grasses are of grown on 12-14 million is are the main pasture es have been found in urope where the species 1, 96). The seed-borne l, along with their hosts, res in the United States um (97, 108). A survey atory of samples from ned endophyte, and the 1% (95). The dominant -31, and most plants are with a low incidence of

nal ryegrass from eight endophyte *A. lolii* (61). incidence of endophyte s showed that only four f in these cultivars (61). on hectares of perennial ommonly sown cultivars e is little information on alian ryegrass pastures. nt cultivars, but in New ultivars may be infected

amorphic state *Acromonium* are symptomless. The t several techniques are ophytes.

STAINING OF MYCELIUM. Grasses infected with endophytes have hyphae in the leaf sheaths. If the adaxial epidermis is removed and stained, the septate, intercellular mycelium can be seen with the aid of a light microscope (16, 55). Hyphae of *Acromonium* spp. and *E. typhina* run parallel to the leaf-sheath axis and are seldom branched, whereas hyphae of the *Gloecladium*-like and *Phialophora*-like endophytes are branched profusely and present in greater quantities than those of the *Acromonium* spp. (59). Most of the endophytes can be found in all above-ground parts of the plant, and hyphae of the *Gloecladium*- and *Phialophora*-like spp. are also present in roots (59). Some endophytes prevent seed formation, but with others, viable seeds are produced that contain a dense mat of mycelium in the region of the aleurone layer (28, 69). This mycelium can be detected by softening seeds with sodium hydroxide or nitric acid, followed by depluming, squashing, and staining for microscopic examination (16). Unfortunately, the technique does not indicate whether mycelium is viable. In order to demonstrate endophyte viability, seeds must be germinated and the 3-4-week-old seedlings examined for mycelium (59, 108). A staining technique that distinguishes between living and dead mycelium in seeds would be of great value.

SEROLOGY. Antisera have been prepared to cultures of *A. coenophialum* (48) and *A. lolii* (72) and used in an enzyme-linked immunosorbent assay (ELISA) to detect antigens of the fungi in tall fescue and perennial ryegrass. ELISA based on antibodies produced against mycelia of these two fungi does not differentiate serologically between *A. coenophialum*, *A. lolii*, *Acromonium*-like spp. in Italian ryegrass, and *E. typhina* (49, 74; D. R. Musgrave, personal communication). Musgrave et al (74) suggest that the major soluble antigen is a polysaccharide moiety of a protein lipopolysaccharide complex. They speculate that purified soluble antigens could be used to produce specific antisera for each of the endophytes. The ELISA technique is particularly useful for determining the location and quantity of mycelium in infected plants and for detecting endophyte mycelium in seeds (44, 72). However, difficulties experienced in using the technique have led to a decrease in its use for endophyte detection, and most endophyte diagnostic laboratories now use the staining technique (95, 108, 109). ELISA does not differentiate between viable and nonviable mycelium, so seed must be germinated and the young plants tested by ELISA or stained to verify the viability of mycelium in seed.

CULTURE. Fungal endophytes of grasses can be cultured on artificial media, and most have been induced to sporulate. In some plant tissues, and especially in seeds, it is difficult to identify endophytes in situ, and so it is necessary to culture the fungi. Many endophytes grow very slowly in culture and are easily overrun by contaminating fungi and bacteria. Hence, culturing endophytes

from infected plant tissues is not used as a routine method of endophyte detection, but rather as an adjunct for endophyte identification. It is relatively easy to isolate and culture endophytes from leaf sheaths, nodes, and stem pith (9, 16, 55, 116), but isolating endophytes from seeds is more difficult, especially from seeds heavily contaminated with saprophytic fungi. The method most commonly used is to surface sterilize seeds in sulfuric acid (59, 108), but even this drastic treatment does not remove all fungi from heavily contaminated seeds. There is evidence (108) that this treatment kills the endophyte in some seeds. Thus, a more satisfactory surface-sterilization procedure is required.

INSECT BIOASSAY When given the choice of endophyte-infected and endophyte-free grass, some species of insects will feed only on the endophyte-free grass (31, 47, 58, 86). A relatively clear cut preference for endophyte-free tall fescue and perennial ryegrass was shown by several species of aphids (47, 58). This finding led Latch et al (58) to advocate the use of *Rhopalosiphum padi* as a bioassay to detect tall fescue plants infected with *A. coenophialum* for large-scale, rapid screening of this endophyte in seedlings and mature plants.

Methods of Control

As previously mentioned, it is thought that endophytes of ryegrass and tall fescue are transmitted only through seed and that infection of endophyte-free grasses does not occur in nature (96, 119). Hence, if endophyte mycelium in seeds can be killed without harming the seed, or if mature infected plants can be freed from endophyte infection, then these plants will remain free of endophyte and will set endophyte-free seed. Several methods of killing endophytes in seeds and plants have been devised.

STORAGE OF SEED Viability of *A. lolii* and *A. coenophialum* mycelium in seed declines during storage (55, 75, 98). Neill (75) found that endophyte in perennial ryegrass seed was dead after two-years storage at ambient temperature, but it was shown subsequently that endophytes in perennial ryegrass seed remained viable for at least fifteen years if stored at low temperature (0-5°C) and low humidity (25-50%) (54). Recent studies have quantified the effect of environmental conditions and demonstrated that low temperature and low seed-moisture content will preserve endophyte viability (88, 108). If it is necessary to kill the endophyte but not greatly harm seed viability, the seed should be stored at high temperatures (30°C) and at a seed-moisture content of 8-10%.

HEAT Soaking seeds of perennial ryegrass and tall fescue in hot water for short periods of time killed the endophytes within them, but seed germination

ne method of endophyte
mination. It is relatively
aths, nodes, and stem pith
seeds is more difficult,
s saprophytic fungi. The
seeds in sulfuric acid (59,
ive all fungi from heavily
t this treatment kills the
story surface-sterilization

endophyte infected and end-
ed only on the endophyte-free
preference for endophyte-
several species of aphids
scale the use of *Rhopalosiphum*
plants infected with *A.*
us endophyte in seedlings

hytes of ryegrass and tall
fection of endophyte-free
if endophyte mycelium in
mature infected plants can
plants will remain free of
veral methods of killing
l.

coenophialum mycelium in
i) found that endophyte in
orage at ambient tempera-
ytes in perennial ryegrass
stored at low temperature
udies have quantified the
d that low temperature and
viability (88, 108). If it is
in seed viability, the seed
a seed-moisture content of

all fescue in hot water for
sem. but seed germination

was reduced to up to 50% (55, 98, 118). A complete kill of endophyte with
only a minor reduction in seed viability was obtained when tall fescue seed
was kept in hot moist air (49°C) for seven days (98).

CHEMICAL. Endophyte mycelium in seeds of perennial ryegrass and tall
fescue has been killed by applying fungicides that inhibit ergosterol biosyn-
thesis, such as propiconazole, prochloraz, and triadimefon, to seeds (55, 98,
119). High application rates of fungicides are required for complete control of
endophytes, and some stunting and death of seedlings are inevitable. Higher
rates of fungicide application are required to kill mycelium of *A. coenophialum*
in seed of tall fescue than those needed for *A. lolae* in perennial ryegrass.
These fungicides have been less effective in controlling endophytes growing
in plants. Repeated foliar applications of triadimefon to plants grown in a
greenhouse reduced the amount of *A. coenophialum* mycelium in tall fescue,
but did not eradicate the fungus (98, 119). Granules of propiconazole applied
to the soil in pastures of tall fescue suppressed the endophyte for many
months, but did not eradicate it (119). Complete eradication of endophyte in
perennial ryegrass and tall fescue has been accomplished by growing plants in
sand and drenching benomyl around the roots (55; G. C. M. Latch, un-
published data), and in tall fescue by using bitertanol or propiconazole
granules and foliar sprays (98, 119). With the fungicides available at present,
it is clear that eradication of endophytes from infected pastures is not economi-
cally feasible. There is, however, a great need for a fungicide that could
inexpensively accomplish this purpose.

Plants and seeds from which endophytes have been eliminated by any of the
three methods just described will produce endophyte-free seed that can then
be used as mother stock to build up endophyte-free lines. Thus, the simplest
and most effective way of controlling animal disorders that result from
grazing endophyte-infected grass is to establish pastures from endophyte-free
seed. In the United States several new cultivars of tall fescue have recently
been released and certified to have fewer than 5% of seeds infected with *A.*
coenophialum (10, 44, 109).

ANIMAL TOXICITIES

As indicated in the introduction, renewed interest in endophyte-infected
grasses began with a few key investigations into causes of animal maladies,
specifically fescue toxicosis and ryegrass staggers (9, 27). Losses due to these
disorders, in terms of animal production, have been estimated to be in the
hundreds of millions of US dollars annually (82; J. A. Boling, personal
communication). However, these estimates do not include losses in reproduc-
tion of cattle that may also be associated with these livestock disorders (36).
Reproductive capacity of both female and male mammals appears to be

decreased on diets high in endophyte-infected forage or seed (77, 104, 121, 123).

Fescue Toxicosis

The poor cattle performance associated with *A. coenophialum*-infected tall fescue is characterized by reduced weight gain, decreased milk production, lower feed intake, rough hair coat, excessive salivation, increased respiration rate, and high rectal temperatures (40). These symptoms most often occur in, but are not limited to, the summer months. An additional malady of cattle associated with endophyte-infected tall fescue in some localities is characterized by lameness, ischemic necrosis, dry gangrene of the extremities, and, in severe cases, loss of hooves (35, 40). This latter malady is known as fescue foot and occurs most frequently during the winter, with the onset of clinical symptoms often linked to a sudden drop in ambient temperature.

Several alkaloids have been suggested to contribute to fescue toxicosis. The loline alkaloids, *N*-acetyl and *N*-formyl, have been shown to reach concentrations as high as 0.8% per unit dry weight in stems, leaves, and seeds of endophyte-infected tall fescue and have not been detected in endophyte-free tall fescue plants (15, 50). It has not been established if the loline alkaloids play a role in fescue toxicosis. However, these alkaloids do appear to serve as insect-feeding deterrents (45, 47).

The ergot alkaloids, clavine and ergopeptide types, are produced *in vitro* by *A. coenophialum* (5, 67, 80) and have been found in low concentrations (relative to the loline alkaloids) in leaf blades and sheaths of endophyte-infected tall fescue collected from pastures (67, 120). Attention has been primarily focused on ergovaline, an ergopeptide alkaloid that constitutes 10–50% of the total ergot alkaloid concentration in the forage (5, 67). Many of the symptoms of fescue toxicosis are consistent with signs of ergot poisoning (5, 68, 120).

Ryegrass Staggers

Perennial ryegrass staggers is a neurological disorder of animals that most commonly affects sheep, but also occurs in cattle, horses, and deer (71). Animals with this disorder appear normal until disturbed, at which time they suffer severe muscular spasms, usually resulting in collapse followed by an apparently quick recovery (71, 97). In severe outbreaks, deaths from falls or drowning are common (71). Ryegrass staggers occurs during the summer and autumn and is commonly associated with warm ambient temperatures and with grazing of short-grass swards (71). In addition to the staggers condition, animals grazing *A. lolii*-infected perennial ryegrass show reduced weight gains, which are probably attributable to reduced intake of herbage (26).

The prime suspect causative agents of ryegrass staggers are the lolitrem

age on seed (34).

coenophialum-infected tall fescue increased milk production, and increased respiration forms most often occur in additional malady of cattle in the localities is characterized by the extremities, and, in malady is known as fescue with the onset of clinical temperature.

to fescue toxicosis. The en shown to reach contents, leaves, and seeds of infected in endophyte-free seed if the loline alkaloids do appear to serve as

s, are produced in vitro by and in low concentrations in sheaths of endophyte-120). Attention has been alkaloid that constitutes the forage (5, 67). Many with signs of ergot poison-

der of animals that most s, horses, and deer (71). arbed, at which time they collapse followed by an eaks, deaths from falls or rs during the summer and mbient temperatures and to the staggers condition, us show reduced weight intake of herbage (26). staggers are the lolitrem

neurotoxins (32, 34). The major lolitrem neurotoxin, lolitrem B, is a lipophilic complex substituted-indole compound with a molecular weight of 685 and formula $C_{26}H_{35}NO_7$ (84). The lolitrems have not yet been reported to be produced by *A. lolii* in culture, but related terpenogenic toxins, penitremis, panthitrems, and aflatrems, are of fungal origin (34).

Toxicities Attributed to Balansia-Infected Weed Grasses

Many warm-season perennial weed grasses infected with species of *Balansia* may possibly be involved with some outbreaks of animal toxicosis (5, 6). Weed grasses parasitized by *Balansia* species include smutgrass (*Sporobolus poiretii*), bloomisedge (*Andropogon virginicus*), lovegrass (*Eragrostis hirsuta*), and panicum (*Panicum anepic*) (5, 6). Bacon et al (5) reported that in the summer months a large percentage of a pasture may consist of a weed grass and that the majority of the weed grass may be infected by *Balansia* spp. Several species of *Balansia* have been shown to produce ergot alkaloids in vitro as well as in vivo (3, 5-8). Furthermore, cultures of *Balansia epichloe* isolated from infected smutgrass depressed serum prolactin concentrations in lactating Holstein cows following oral dosing for three consecutive days (107). Prolactin is a pituitary hormone that is necessary for the secretion of milk and that probably also plays a role in affecting the reproductive capacity of both female and male mammals (107). Depressed serum prolactin levels also occur in animals that have consumed *A. coenophialum*-infected tall fescue forage (12, 40, 42) as well as in sheep that have grazed *A. lolii*-infected perennial ryegrass (26).

GRASS-FUNGUS INTERACTIONS

Parasitic and Mutualistic Symbiosis

The endophytic fungi infecting grasses are biotrophic; that is, they obtain all their basic nutritional needs from living tissue. Furthermore, Lewis (62) considers fungi such as *E. typhina* and those responsible for "symptomless parasitism" as belonging to a group of ecologically obligately symbiotic biotrophs (obligate biotrophs). Lewis defined this group of fungi as "parasitic and mutualistic symbionts with no capacity for free living existence other than as gametes, cysts or as laboratory cultures." The terminology and classification are based on de Bary's (22) original concept of symbiosis: "Those which . . . feed on living organisms, whether plants or animals, are termed parasites. Their relationship with their hosts is that of a common life, a *symbiosis*" de Bary recognized that gradation of symbiosis existed and that parasites could either quickly destroy their host or that parasite and host could mutually and permanently live together and support one another.

According to Lewis's scheme, *E. typhina* and *Balansia* spp. would be

considered biotrophic and parasitic endophytes, whereas the *Acremonium* endophytes would be biotrophic and mutualistic symbionts. This scheme of classification of *E. typhina* is not absolute because isolates in some host grasses show gradations of symbiosis. Some isolates act as parasitic symbionts (24, 130), whereas others behave, e.g. the symptomless *Acremonium* endophytes, as mutualistic symbionts (29, 92). Whether a specific symbiotic association is parasitic or mutualistic can only be determined by comparing the fitness of the host and fungus when living independently with their fitness when living in association (63).

The major features of mutualistic symbioses are a lack of destruction of host cells or tissue, nutrient or chemical cycling between host and fungus, enhanced longevity and photosynthetic capacity of cells and tissue under the influence of infection; enhanced survival of the fungus; and a tendency toward greater host specificity than seen in necrotrophic infections (62). Researchers (5, 10, 82, 96, 97) have used this concept of mutualism to suggest that the *Acremonium* endophytes infecting perennial ryegrass and tall fescue enter into a relationship with their respective grasses that benefits both host and fungus. These benefits include enhanced dissemination and survival of the fungus, improved growth for the host plant, and tolerance to herbivore feeding (grazing animals and insects).

Benefits to the Fungus

Detection of endophytes by electron microscopy (25, 41), staining (9, 23, 108), and ELISA (72, 96) clearly indicates that intercellular fungal growth of *Acremonium* spp. and isolates of *E. typhina* is distributed unequally in the plant, with highest concentrations found in the leaf sheaths, seeds, and crowns. These regions can act as "sinks" for the accumulation of soluble nitrogenous and carbohydrate substrates (5). These substrates would have to leak out of the plant cells into the intercellular spaces and fluids for uptake by the endophytes. Whether the fungi induce leakage or possess translocational mechanisms for movement of plant products is unknown. However, reciprocal translocation of carbohydrates between host and pathogen has been reported in Bahia grass infected with *Myriogenospora atramentosa* (99). The nutritional requirements of the fungi in the plant are unknown.

In vitro rates of growth, pH and temperature optima, utilization of carbon and nitrogen sources, and synthesis of specific fungal products are known for some endophytes (3, 9, 20, 53, 59, 114). This information cannot be extrapolated to the in vivo situation, but can be used to compare nutritional, physiological, biochemical, and genetic potential of isolates (10). Clearly, the endophytes benefit from the association with their hosts by nutrition, long-term protection, improved dissemination (via seed), and survival.

heras the *Acremonium* rhizoids. This scheme of re isolates in some host ex act as parasitic symptomless *Acremonium* that a specific symbiotic determined by comparing identifi with their fitness

a lack of destruction of tween host and fungus cells and tissue under the s, and a tendency toward ctions (62). Researchers ctions to suggest that the and tall fescue enter into its both host and fungus. survival of the fungus, re to herbivore feeding

5, 41), staining (9, 23, cellular fungal growth of tributed unequally in the raf sheaths, seeds, and accumulation of soluble substrates would have to and fluids for uptake by r possess translocational own. However, recipro- f pathogen has been re- *atramentosa* (99). The re unknown. ma, utilization of carbon l products are known for 'ormation cannot be ex- to compare nutritional, solates (10). Clearly, the nosts by nutrition, long- and survival.

Benefits to the Host

GROWTH Perhaps of all the potential benefits to infected grasses, changes in plant physiology and patterns of growth are the most complex and difficult to assess. In studies in Kentucky over a four-year period, Siegel et al (96) did not find differences in forage (dry matter), seed yield, and stand persistence of Kenia tall fescue in plots that ranged from 7-75% of plants infected with *A. coenophialum*. On the other hand, there are reports suggesting that endophyte-free cultivars do not survive as well as infected ones. In Texas, infected tall fescue that had been grazed produced more forage than plants in noninfected grazed pastures (87). In addition, during the third year of the trial many plants died in two of the three noninfected replicated plots. Funk et al (29) reported that over a seven-year period in New Jersey, endophyte-infected tall fescue turf plants outperformed noninfected turf plants from the standpoint of resistance to crabgrass invasion and recovery from summer drought stress.

The differences in the three reports are difficult to reconcile, but as will be shown, infected perennial ryegrass and tall fescue exhibit striking resistance to herbivores (insects and grazing animals). A combination of environmental stresses (heat and drought), herbivore feeding, and competition with other grass and weed species could account for the differences reported in growth and survival between infected and noninfected grasses. Biotic and abiotic stresses have been reported to be the reason for the inability of endophyte-free perennial ryegrass cultivars to survive in New Zealand (11, 82).

There are no differences in forage quality (crude protein, neutral detergent fiber, acid detergent fiber, and digestibility) between endophyte-infected and noninfected tall fescue (14). When infected tall fescue was grown under controlled conditions, the plants had greater rates of photosynthesis and a greater increase in fresh weight; they produced more tillers during regrowth, and they had a lower percentage leaf roll (during a drying cycle) than did endophyte-free tall fescue. Infected plants also used water more efficiently than did noninfected plants (L. P. Bush, personal communication). These differences would support the hypothesis that infected tall fescue plants have advantages over noninfected plants when grown during periods of environmental stress.

The clearest example of enhanced growth has been reported for *A. lolae*-infected perennial ryegrass grown under controlled conditions in New Zealand (60). Infected plants (when compared to noninfected) had significant increases in total leaf area, tiller numbers, and growth of leaves, but the shoot-root ratio was unaffected.

The chemical basis for the changes in physiology and growth patterns of *Acremonium*-infected plants is unknown. It is possible that members of the

Table 1. Species of insects reported to be affected by endophyte-infected *Festuca* and *Lolium* species of grasses^a

| Insect | Grass | Fungus | Reference |
|----------------------------------------------------------|-----------------------------------------------|---------------------|-----------|
| <i>Crambus</i> spp. (Sod webworms) | PRG ^b | A | 61 |
| <i>Lophoceros cornutus</i> (Acyrthos Stem Weevil) | PRG, TI ^c | A, A c | 86, 101 |
| <i>Sphenophorus putrescens</i> (Bluegrass billbug) | PRG ^b | A c | 2 |
| <i>Spodoptera frugiperda</i> (Fall armyworm) | PRG, TI | A, A c | 38, 39 |
| <i>Acheta domestica</i> (House cricket) | PRG, TI | A, A c | 1 |
| <i>Rhopalosiphum padi</i> (Oat bird cherry aphid) | TI | A c | 47, 58 |
| <i>Schizaphis graminum</i> (Greenbug aphid) | PRG, TF, HF ^d , CT ^e | A, A c, T c, E c | 47 |
| <i>Onoscorphus fasciatus</i> (Milkweed bug) | TI | A c | 47 |
| <i>Blattella leucophaea</i> (Crunch bug) | CT ^e | E c | 29 |
| <i>Stethorus punctatus</i> (Black beetle) | PRG ^b | A c | 82 |
| <i>Oraculacephala antia</i> (Sharpshooter leafhopper) | TF ^b | A c | 52 |
| <i>Chaetocnema pulicaria</i> (Corn flea beetle) | TF ^b | A c | 52 |

^a Abbreviations: TF, tall fescue (*F. arundinacea*); PRG, perennial ryegrass (*L. perenne*); CT, Chewings fescue (*F. rubra* subsp. *commutata*); HF, hard fescue (*F. longidolus*); A c, *A. coenophialus*; A, *A. lolae*; L, *L. typhina*.

^b Based on field observations.

^c M. Siegel, unpublished data.

Clavicipitaceae produce, or induce the plant to produce, auxinlike plant-growth regulators and/or plant growth inhibitors, or they may alter hormone metabolism. These are distinct possibilities when one considers that symptoms of infection by *E. typhina*, and other members of the Balansiae, include not only the inhibition of flowers and seed, but also enhanced plant growth and tillering, dwarfism, or deformation of the flag leaf (5, 17, 24, 37, 60, 110). Auxinlike indole compounds have been isolated in vitro from cultures of *Balansia epichloae* (79). The production of these compounds may reflect an alteration of nitrogen metabolism and assimilation in the infected plant (5).

TOLERANCE TO INSECT HERBIVORES Perhaps the most striking difference between *Acremonium*-infected and noninfected *Festuca* and *Lolium* species of grasses is the resistance by infected grasses to attack by insects (Table 1). It is

or endophyte infection

as Reference

A. 80 10

A. 09 3

A. 4 88

A. 17

(F) 17

24

87

52

52

ryegrass *L. perenne*
and *L. multiflorus*, A.

produce, auxinlike plant
they may alter hormone
ne considers that symp-
of the Baiansiae, include
enhanced plant growth
leaf (5, 17, 24, 37, 60,
ed in vitro from cultures
compounds may reflect an
in the infected plant (5).

most striking difference
ca and *Lolium* species of
by insects (Table 1). It is

now known that *Acetmonium*-infected grasses produce an array of chemicals that have a wide range of biological activity (5, 10, 14, 82, 97).

The compounds responsible for insect (Argentine stem weevil, *Listronotus bonariensis*) resistance in *A. lolii*-infected perennial ryegrass have been identified as a peramine alkaloid (89) and the lolitrem neurotoxins (84). Peramine (89, 90), or a peramine-like compound (85), is made in culture by *A. lolii*.

The ergopeptide alkaloids (predominately ergovaline) isolated from *A. coenophialum*-infected tall fescue (67, 120) have not been reported active against insects. However, clavine ergot alkaloids (ergotamine and ergonovine) do have insecticidal properties (D. L. Dahlman, personal communication). The pyrrolizidine alkaloids (*N*-formyl and *N*-acetyl loline) produced in *A. coenophialum*-infected tall fescue (13, 15, 43, 50, 51) have also been reported responsible for insect resistance (45, 47). These alkaloids have not been recovered from either mycelium grown in culture or viable fungal mats isolated from enzyme-digested seed (L. P. Bush, personal communication). A steroid tetraenone metabolite (ergosta-4,6,8-(14), 22-tetraen-3-one), biologically active in brine shrimp and chick embryo bioassays, has been isolated from infected tall fescue and cultures of *A. coenophialum* (9, 21). This compound has not been tested for activity against insects.

Techniques now exist for producing synthetic grass-endophyte complexes. This synthesis involves the transfer of different species of fungi to host grasses and grasses not known to be hosts by inoculation of one-week-old seedlings (via wounding of the meristem) (56), by inoculation of callus tissue (46), and by plant breeding (maternal-line selection) (29, 30). These techniques should assist in further characterization of the grass-endophyte relationship and biological activity of synthesized chemicals.

One may assume that if biologically active chemicals are of fungal origin, they could be produced in any naturally occurring as well as in synthetic grass-endophyte combinations. Conversely, if compounds are only produced in conjunction with certain grass-fungus interactions, then a greater degree of specificity of synthesis would be expected. Peramine is not only produced in *A. lolii*-infected perennial ryegrass, but in *A. coenophialum* and *E. typhina* naturally infected grasses as well (M. R. Siegel, unpublished data). In addition, peramine was found in two synthetic grass-endophyte combinations: an *A. lolii*-infected tall fescue and *A. coenophialum*-infected perennial ryegrass. There appears to be a much greater specificity of synthesis of the loline alkaloids in infected grasses. Synthesis occurred only in *A. coenophialum*-infected tall fescue and in the synthetic *A. coenophialum*-infected perennial ryegrass. These alkaloids could not be recovered from tall fescue artificially infected with *E. typhina*, *A. lolii*, or *Phialophora*-like endophytes. Loline alkaloids have been reported in *L. temulentum* (19);

however, it is not known whether the plant material was infected with the *Acremonium* like species that has been isolated from this grass (87). These data still do not explain whether the host, fungus, or both are responsible for synthesis of the loline alkaloids; it has yet to be determined whether the ergot alkaloids and lolitrems can be produced by other fungi on their natural hosts or by the fungi in nonhost grasses.

The ability of endophyte-infected plants to produce biologically active compounds under field conditions may depend on the location and concentration of the endophytes in the plant. Synthesis of these compounds may depend on factors that affect plant and fungal growth. These include internal and external nutritional conditions and the external environment (light, water, and temperature). The fungus-produced toxins peramine (90), ergot alkaloids (5, 67, 81), and lolitrems (33, 34) (classified here as being of fungal origin because of their similarity to other mycotoxins) are found in relatively low concentrations (0.3–22 $\mu\text{g/g}$ plant dry weight) in infected plants. On the other hand, the loline alkaloids, presumed to be of plant origin, are found in concentrations as high as 8,253 $\mu\text{g/g}$ plant dry weight (80).

The distribution and concentration of the chemicals within the plant vary with the compound, the location of the endophyte in the plant, and the season. There is little information on peramine, other than that concentrations of 1 $\mu\text{g/g}$ plant fresh weight were recovered from *A. lolii*-infected plants (89). Leaf sheaths and blades of *A. lolii*-infected perennial ryegrass contained lolitrems at concentrations as high as 4.9 and 0.37 $\mu\text{g/g}$ plant dry weight, respectively (33). The amount of *A. lolii* mycelium in infected plants has been positively correlated with resistance to Argentine stem weevil and with the time of year when ryegrass staggers occurs (11, 27, 82, 83).

Total ergot alkaloid concentrations in leaf sheaths and blades of *A. coenophialum*-infected tall fescue plants were 22 and 3.5 $\mu\text{g/g}$ plant dry weight, respectively (5, 67). Total ergot alkaloids increased 2.5-fold in infected tall fescue following application of nitrogen fertilizers (67). Furthermore, added nitrogen was associated with increased growth of endophyte in the plant and increased symptoms of animal toxicoses (67). High levels of loline alkaloids have been found in the same parts of the plant where high densities of the fungus are known to exist (13, 43, 50, 51). In greenhouse studies, the fungus concentration in the plant did not increase during initial growth, but increased in first and second regrowth tissues (13). Loline alkaloid concentrations followed the same pattern, except after the second harvest, when concentrations in the blade (where there was little fungus) nearly equalled those in the stem. This finding suggests that these alkaloids, or the signal for their synthesis, are translocated to the leaf blades, and hence, regrowth tissue in the field may contain large amounts of alkaloids. In greenhouse experiments, loline alkaloids increased 2–3-fold in plants sub-

it was infected with the grass (57). These both are responsible for mined whether the ergot is in their natural hosts or

duce biologically active ergot alkaloids and congeneric compounds may depend on environmental factors including light, water, and temperature (58). Ergot alkaloids (59) and fungal origin are found in relatively low infected plants. On the other hand, ergot alkaloids are found in high (50).

als within the plant vary with the plant, and the season that concentrations of *L. loliae* infected plants (89). Annual ryegrass contained 7 $\mu\text{g/g}$ plant dry weight. In infected plants has been stem weevil and with the 7, 82, 83).

aths and blades of *A. coenophialum* and 3.5 $\mu\text{g/g}$ plant dry weight increased 2.5-fold in fertilizers (67). Further growth of endophyte in grasses (67). High levels of the plant where high (50, 51). In greenhouse not increase during initial with tissues (13). Loline, except after the second there was little fungus. It is suggested that these alkaloids, the leaf blades, and hence, amounts of alkaloids. In 12-3-fold in plants sub-

jected to moderate-to-severe water stress (51). However, in contrast to the ergot alkaloids, increased application of nitrogen did not increase levels of loline alkaloids (51). Concentrations of loline alkaloids were monitored in tall fescue during a three-year steer grazing study (103). They generally rose to their highest concentrations in August, and then remained constant until late fall. However, concentrations varied greatly from year to year, suggesting an effect of environment on alkaloid concentration in the plant.

There is little information on the effect of concentration of chemicals in infected plants and insect toxicity. Using the large Milkweed bug (*Oncopeltus fasciatus*) bioassay (47), D. L. Dahman (personal communication) has found 10% concentrations of ca 8 $\mu\text{g/m}$ for N-formyl loline, ergotamine, and ergonovine. Agar-plate bioassays of peramine with Argentine stem weevil indicated that feeding deterrence occurred at 10 $\mu\text{g/ml}$ (89). No feeding deterrence occurred with lolitrems, but after 24-days feeding on 5 $\mu\text{g/ml}$, larval weights were reduced and 40% mortality occurred (84).

TOLERANCE TO GRAZING HERBIVORES In the section on animal toxicoses, we discussed the relationship between endophyte-infected grasses and animal maladies. One of the direct effects on animals grazing endophyte-infected grasses is a reduction in average daily weight gains. Of equal importance is the observation that infected tall fescue is tolerant to overgrazing by livestock (J. A. Boling, personal communication). The lack of overgrazing of the infected cultivars may be due to better growth of the plants and differences in agronomic factors between the infected and noninfected cultivars. More likely, it is due to the fact that animals feeding on infected perennial ryegrass and tall fescue consume substantially less forage than those feeding on noninfected grass (26, 102).

DISEASE RESISTANCE The last of the benefits of endophyte-infected grasses is the potential for disease resistance. White & Cole (113, 114) and M. R. Siegel & G. C. M. Latch (unpublished data) have demonstrated that isolates of *A. lolii*, *A. coenophialum*, unidentified *Acremonium* sp., *Phialophora*-like sp., and *E. typhina* from various grass spp. produced antibiosis against a range of fungal plant pathogens and saprophytic fungi in culture. The amount of antifungal activity and the spectrum of activity were found to be dependent on the isolate. Resistance to diseases in pasture or turf *Festuca* and *Lolium* spp. has not been reported.

Speculations on the Origins of Grass Endophytes

The recent origins of *A. coenophialum* and *A. lolii* infected pasture and turf grasses have been discussed elsewhere (10, 96, 97). It is clear that Europe is the origin of these infected cultivated grasses. However, the determination

that endophytes not only exist in native *Festuca* and *Lolium* species (112, 114, 116), but in large numbers of species in the Poaceae (111, 115, 117) as well. It raises important questions concerning the recent origins of these endophytes and also the evolutionary relationships between grasses and endophytes. Because of the wide geographic distribution of cultivated and native grasses infected with endophytes, White (174) has suggested that the vegetative phases of these fungi are, or have evolved from, species of the ascomycetous genus *Epichloe*. Mycological and serological data (49, 73) and evolutionary concepts of biotrophy (63) give some support to this hypothesis.

Lewis (63) considers the biotrophic habit of great antiquity and believes that the basic features of the evolution of parasitism and mutualism within biotrophy occurred at least 400 million years ago. The evolution from parasitism to mutualism therefore depends on the capacity of the partners to avoid or tolerate environmental stresses, as well as the obvious capacity for two-way cycling of nutrients and other prerequisites of biotrophy previously discussed. As was pointed out earlier, *E. typhina* appears to be in the process of evolution, as indicated by the various degrees of symbiosis that exist between different isolates and their hosts. The end result of this evolutionary process would then be fungi like the *Acremonium* spp., which act as mutualistic symbionts.

USE OF ENDOPHYTE-INFECTED AND NONINFECTED GRASSES

Once specific aspects of the nature of the host-fungus interaction were recognized, then the advantages of both infected and noninfected grasses were realized. The fact that the grass-endophyte relationship could be manipulated to remove or introduce fungi into different cultivars of grasses only served to accelerate their use. However, it is now apparent from a better understanding of the grass-fungus relationship that whereas some uses of endophyte-free cultivars are desirable, they may not be practical. Instead, further manipulations of infected grasses may be necessary to realize the full potential of improved productivity from animals grazing infested pastures.

Use of Endophyte Free Cultivars

As soon as it was recognized that endophyte-infected grasses were responsible for animal toxicoses, it was suggested that removal of the fungi would lead to an alleviation of symptoms and improved animal productivity (9, 27). Eradication of *A. lolii* and *A. coenophialum* from perennial ryegrass and tall fescue cultivars, respectively, was accomplished by killing the fungi in plants or seed by storage, heat, or chemical treatments.

Some of the endophyte-free tall fescue cultivars were clearly superior to

and *Lolium* species (112, 113) as recent origins of these relationships between grasses and endophytes of cultivated and wild grass species of the tropical data (49, 73) and support to this hypothesis of antiquity and believes in mutualism within the evolution from parasitism of the partners to avoid our capacity for two-way phy previously discussed to be in the process of mutualism that exist between this evolutionary process which act as mutualistic

3. NONINFECTED

fungus interaction were noninfected grasses were manipulated grasses only served to on a better understanding ie uses of endophyte-free Instead, further manipulate the full potential of ted pastures

d grasses were responsible of the fungi would lead to al productivity (9, 27). Perennial ryegrass and tall killing the fungi in plants

s were clearly superior to

infected cultivars from the standpoint of improved animal production (93, 102). However, the endophyte-free perennial ryegrass cultivars used in pastures in New Zealand were susceptible to insect predation and drought, and often did not survive (11, 82). Whether the same phenomenon will occur with the newly released endophyte-free tall fescue cultivars is unknown and is a topic of considerable speculation.

What is unclear is whether the superior character of tall fescue is due to the endophytic grass complex or, in part, to the normal genetic complement of the plant. Consequently, it may be necessary to sow large areas of endophyte-free pastures before an appropriate evaluation of survival can be made.

Use of Endophyte-Infected Grasses

The confirmation of insect resistance, enhanced growth, and persistence of endophyte-infected grasses has led grass breeders to develop, by maternal line selection, naturally infected cultivars for turf (29-31). Seed of infected grasses used in turf will require special storage facilities and labeling to guarantee the viability of the endophytes.

Another approach to the use of endophyte-infected grasses would be to introduce specific fungal isolates, via wounding of seedlings, into host or nonhost grasses (synthetic grass-endophyte combinations). Assuming that the compounds toxic to animals are different from those that affect insects, it should be possible to develop synthetic combinations of host and endophyte suitable for turf and pasture. Insect deterrent compounds would be present in both turf and pasture cultivars. Turf grasses could still contain compounds toxic to animals, but pasture grasses would have little or none (10, 83, 89). Endophytes in these synthetic combinations could be naturally occurring biotypes, if suitable ones were available, or they could be genetically modified. All endophytes must, of course, be seed transmitted.

We have made a number of positive, but unproven, assumptions to support the use of modified endophyte-infected pasture grasses. We already know that the grass-endophyte system in some cases may contain more than one chemical responsible for insect resistance. This fact raises the question of what happens to insect resistance when one of several toxic compounds is not present in the modified synthetic combinations. It is obvious that it will be necessary to identify all the biologically active compounds and their spectrum of activity in plants, insects, and animals before new synthetic grass-endophyte cultivars are released for pasture use.

CONCLUDING REMARKS

Through interdisciplinary research, plant pathologists, chemists, agronomists, entomologists, and animal scientists have come to better understand a

unique grass-fungal endophyte relationship. It is now clear that in certain grass-fungus complexes, mutualistic relationships result in benefits to both partners. Understanding this relationship has resulted in the development and release of both infected and noninfected cultivars for use in pasture and turf.

In order to ensure the proper use of these newly developed cultivars, interdisciplinary research must continue into the nature of the grass-endophyte relationship. Questions of interest and importance that need to be answered include the mechanisms of synthesis and the spectrum of activity of biologically active compounds; the interaction of external and internal stimuli on synthesis of these chemicals and on growth of the endophytes and plants; biochemical mechanisms of pest resistance and animal toxicoses; and the nature of biotrophy and how it relates to the ecological fitness and survival of the endophytes and grass hosts.

The widespread distribution of fungal endophytes in grasses and the potentially mutualistic nature of the relationships may suggest an importance that approaches other plant-microorganism complexes such as those that involve the mycorrhizal fungi and nitrogen-fixing bacteria. The improvement of grasses used for pasture, turf, and conservation are long-term goals of plant breeders; a fuller understanding of grass-endophyte relationships will aid in fulfilling these goals.

ACKNOWLEDGMENT

We wish to thank many authors for providing unpublished information and copies of manuscripts in press. Research in the senior author's laboratory was supported, in part, by USDA Competitive Research Grant 85-CRC-1-1533. This chapter is Kentucky Agricultural Experiment Station Journal Series Paper 87-11-9.

Literature Cited

- Ahmad, S., Govindarajan, S., Funk, C. R., Johnson-Cicalese, J. M. 1985. Fatality of house crickets on perennial ryegrass infected with a fungal endophyte. *Entomol. Exp. Appl.* 39:183-90.
- Ahmad, S., Johnson-Cicalese, J. M., Dickson, W. K., Funk, C. R. 1986. Endophyte-enhanced resistance in perennial ryegrass to the bluegrass bill-bug *Sphenophorus parvulus*. *Entomol. Exp. Appl.* 41:7-10.
- Bacon, C. W. 1985. A chemically defined medium for the growth and synthesis of ergot alkaloids by species of *Balanus*. *Mycologia* 77:418-23.
- Bacon, C. W., Hinton, D. 1983. Biology of the endophyte of fescue: Ultrastructural analysis and physiological relationships. In *Proc. Forage & Turf Grass Endophyte Workshop*, pp. 19-28. Corvallis, Ore: Oregon State Univ.
- Bacon, C. W., Lyons, P. C., Porter, J. K., Robbins, J. D. 1986. Ergot toxicity from endophyte-infected grasses. A review. *Agron. J.* 78:106-16.
- Bacon, C. W., Porter, J. K., Robbins, J. D. 1975. Toxicity and occurrence of *Balanus* on grasses from toxic fescue pastures. *Appl. Microbiol.* 29:553-56.
- Bacon, C. W., Porter, J. K., Robbins, J. D. 1979. Laboratory production of ergot alkaloids by species of *Balanus*. *J. Gen. Microbiol.* 113:119-26.
- Bacon, C. W., Porter, J. K., Robbins, J. D. 1981. Ergot alkaloid biosynthesis by

now clear that in certain result in benefits to both of the development and use in pasture and turf wly developed cultivars, e nature of the grass, importance that need to be the spectrum of activity of ternal and internal stimuli e endophytes and plants, nimal toxicoses, and the cal fitness and survival of

in grasses and the poten- agest an importance that such as those that involve a. The improvement of long-term goals of plant e relationships will aid in

ublished information and or author's laboratory was h Grant 85-CRC-1-1533. nt Station Journal Series

ural analysis and physiological daps. In *Proc. Forage & Turf endophyte Workshop*, pp. 19-26 s, Ore. Oregon State Univ. C. W., Lyons, P. C., Porter, J. ums, J. D. 1986. Ergot toxicity dephyte-infected grasses. *A. regon. J.* 78:106-16. W., Porter, J. K., Robbins, J. e. Toxicity and occurrence of n grasses from toxic fescue. *Appl. Microbiol.* 29:553-56. W., Porter, J. K., Robbins, J. Laboratory production of ergot y species of *Balarium*. *J. Gen. et.* 113:119-26. W., Porter, J. K., Robbins, J. Ergot alkaloid biosynthesis by

- isolates of *Balarium epiphyllum* and *B. Aen nigricans*. *Can. J. Bot.* 59:24-32.
10. Bacon, C. W., Porter, J. K., Robbins, J. D., Lunnell, E. S. 1977. *Lophosiphium* from toxic tall fescue grasses. *Appl. Environ. Microbiol.* 34:576-81.
11. Bacon, C. W., Siegel, M. E. 1987. The endophyte of tall fescue. *J. Prod. Agr.* In press.
12. Barker, G. M., Prestidge, B. A., Pottinger, R. F. 1985. Strategies for Argentine stem weevil control: Effects of drought and endophyte. *Proc. 5. 2. Grasses*. *Assoc.* 47:107-14.
13. Bush, D. J., Bone, J., Finch, G. P., Elsworth, J. 1987. Concentrations of PHE, LH, FSH, GH and TSH in plasma and pituitary of ewes grazing tall fescue and lucerne/grass pastures. *J. Anim. Sci.* 65(Suppl. 1):44Abstr.
14. Burton, W., Bush, L. P. 1987. Development of the endophyte *Acremonium coenophialum* and accumulation of N-acetyl and N-formyl loline in tall fescue (*Festuca arundinacea* Schreb.). *Crop Sci.* In press.
15. Bush, L. P., Burton, P. H. H. 1987. Tall fescue quality as affected by *Acremonium coenophialum*. *J. Prod. Agr.* In press.
16. Bush, L. P., Cornelius, P. L., Buckner, R. C., Varney, D. R., Chapman, R. A., et al. 1987. Association of N-acetyl loline and N-formyl loline with *Epichloe typhina* in tall fescue. *Crop Sci.* 22:94-43.
17. Clark, E. M., White, J. F., Pauersson, R. M. 1983. Improved histochemical techniques for the detection of *Acremonium coenophialum* in tall fescue and methods of in vitro culture of the fungus. *J. Microbiol. Methods* 1:149-55.
18. Clay, K. 1984. The effect of the fungus *Akissoneella* (Clavicipitaceae) on the reproductive system and demography of the grass *Danthonia spicata*. *New Phytol.* 98:165-75.
19. Cole, G. T., White, J. F. 1985. Fungal endophytes of forage grasses. *Proc. Indian Acad. Sci. (Plant Sci.)* 94:129-36.
20. Culvenor, C. C. 1973. Alkaloids. In *Chemistry and Biochemistry of Herbage*, ed. G. W. Butler, R. W. Bailey, 1:375-446. London-New York: Academic.
21. Davis, N. D., Clark, E. M., Schrey, K. S., Diener, U. L. 1986. In vitro growth of *Acremonium coenophialum*, an endophyte of toxic tall fescue grass. *Appl. Environ. Microbiol.* 52:888-91.
22. Davis, N. D., Coie, R. J., Diener, J. W., Weese, J. D., Backman, P. A., et al. 1986. Steroid metabolites of *Acremonium coenophialum*, an endophyte of tall fescue. *J. Agric. Food Chem.* 34:105-8.
23. de Bary, A. 1887. *Comparative Morphology and Biology of the Fungi, Mycetozoa and Bacteria*. Oxford: Clarendon (151 pp., translation).
24. de Menis, M. E., Waller, J. E. 1969. Visual assessment of seasonal changes in amount of mycelium of *Acremonium coenophialum* in leaf sheaths of perennial ryegrass. *N. Z. J. Agric. Res.* 29:111-16.
25. Diehl, W. W. 1950. *Balarium* and the *Balarium* in America. *Agric. Monogr.* 4. Washington, DC: US Dep. Agric.
26. Fierchter, L. R., Harvey, I. C. In press. Usual crystalline and appropriate of tubules in the *Lolium* endophyte of ryegrass leaf sheaths. *Phytoplasma* 147:17-23.
27. Fierchter, L. R., Barrell, G. K. 1984. Reduced liveweight gains and serum prolactin levels in hogsheads grazing ryegrass containing *Lolium* endophyte. *N. Z. J. Vet. J.* 32:129-40.
28. Fierchter, L. R., Harvey, I. C. 1981. An association of a *Lolium* endophyte with ryegrass staggers. *N. Z. J. Vet. J.* 29:185-86.
29. Freeman, E. M. 1903. The seed-fungus of *Lolium temulentum* L., the darnel. *Phylos. Trans. R. Soc. London Ser. B* 196:1-27.
30. Funk, C. R., Halisky, P. M., Ahmad, S., Hurley, R. H. 1985. How endophytes modify forage performance and response to insect pests in perennials breeding and evaluation trials. In *Proc. 5th Int. Turf Res. Conf.* Avignon, ed. J. Lemaire, pp. 137-45. Versailles, INRA.
31. Funk, C. R., Halisky, P. M., Hurley, R. H. 1983. Implications of endophyte fungi in breeding for insect resistance. *See Ref. 4*, pp. 67-75.
32. Funk, C. R., Halisky, P. M., Johnson, M. C., Siegel, M. E., Stewart, A. V., et al. 1983. An endophyte fungus and resistance to sod webworms. Association in *Lolium perenne* L. *BioTechnol.* 1:189-91.
33. Gallagher, R. T., Hawkes, A. D. 1985. Estimation of neurotoxin levels in perennial ryegrass by mouse bioassay. *N. Z. J. Agric. Res.* 28:272-31.
34. Gallagher, R. T., Hawkes, A. D., Stewart, J. M. 1985. Rapid determination of the neurotoxin lolitrem B in perennial ryegrass by high-performance liquid chromatography with fluorescence detection. *J. Chromatogr.* 321:217-26.

44. Gallagher, R. T., Hawkes, A. D., Steyn, F. S., Viegman, R. 1984. Fungicidal neurotoxins from perennial ryegrass causing ryegrass staggers disorder: Toxicity, structure and elimination of oltiprin by *C. nem. b.* *Chem. Commun.* 614-16.
45. Garner, G. B., Connell, C. N. 1978. Fescue toxicosis in *Micromys funge. Muscivora*. *Musculovora*. *An. En. Lepidoptera Handbook*, ed. T. D. Wells, I. G. Morthouse, 245-62. New York: Dekker.
46. Gay, N., Buling, J. A., Miskal, D. 1986. Effects of fescue pastures on cow calf performance. *J. Anim. Sci.* 63 (Suppl. 1173) (Abstr.).
47. Harberd, D. J. 1961. Note on choke disease in *Festuca rubra*. *Scottish Plant Breeding Station Report* pp. 47-51. Edinburgh: Blackwood.
48. Hardy, T. N., Clay, K., Hammond, A. M. Jr. 1985. Fall armyworm (Lepidoptera: Noctuidae): A laboratory bioassay and larval preference study for the fungal endophyte of perennial ryegrass. *J. Econ. Entomol.* 78:571-75.
49. Hardy, T. N., Clay, K., Hammond, A. M. Jr. 1986. Leaf age and related factors affecting endophyte-mediated resistance to fall armyworm (Lepidoptera: Noctuidae) in tall fescue. *Environ. Entomol.* 15:1085-89.
50. Hemken, R. W., Jackson, J. A. Jr., Boling, J. A. 1984. Toxic factors in tall fescue. *J. Anim. Sci.* 58:1011-16.
51. Hinton, D. M., Bacon, W. C. 1985. The distribution and ultrastructure of the endophyte of toxic tall fescue. *Can. J. Bot.* 63:36-42.
52. Hurley, W. L., Convey, E. M., Leung, K., Ligerton, L. A., Hemken, R. W. 1981. Bovine prolactin, TSH, T₃ and T₄ concentrations as affected by tall fescue summer toxicosis and temperature. *J. Anim. Sci.* 51:374-79.
53. Jackson, J. A., Hemken, R. W., Boling, J. A., Hamon, R. J., Buckner, R. C., Bush, L. P. 1984. Loline alkaloids in tall fescue hay and seed and their relationship to summer fescue toxicosis in cattle. *Dairy Sci.* 67:104-5.
54. Johnson, M. C., Anderson, R. L., Kryscio, R. J., Siegel, M. R. 1983. Sampling procedures for determining endophyte in tall fescue seed lots by ELISA. *Phytopathology* 73:1406-9.
55. Johnson, M. C., Bush, L. P., Siegel, M. R. 1986. Aphid feeding deterrence associated with pyrrolizidine alkaloids present in endophyte-infected tall fescue. *Phytopathology* 76:1057 (Abstr.).
56. Johnson, M. C., Bush, L. P., Siegel, M. R. 1986. Infection of tall fescue with *Acremonium coenophialum* by means of sowing culture. *Plant Dis.* 70:880-82.
57. Johnson, M. C., Johnson, D. L., Siegel, M. R., Bush, L. P., Latch, G. C. M. et al. 1985. Insect feeding deterrents in endophyte-infected tall fescue. *Appl. Environ. Microbiol.* 49:548-73.
58. Johnson, M. C., Plone, J. P., Siegel, M. R., Varrus, D. R. 1987. Detection of *Epichloe typhina* in tall fescue by means of enzyme-linked immunosorbent assay. *Phytopathology* 77:647-50.
59. Johnson, M. C., Siegel, M. R., Schmidt, B. A. 1985. Seasonal activities of endophyte fungi from tall fescue and perennial ryegrass and of *Epichloe typhina*. *Plant Dis.* 69:200-2.
60. Jones, T. A., Buckner, R. C., Burras, P. B. H., Bush, L. P. 1983. Accumulation of pyrrolizidine alkaloids in benomyl-treated tall fescue parents and their untreated progenies. *Crop Sci.* 23:1135-40.
61. Kennedy, C. W., Bush, L. P. 1983. Effect of environmental and management factors on the accumulation of N-acetyl- and N-formyl loline alkaloids in tall fescue. *Crop Sci.* 23:547-52.
62. Korfman, G. W., Brandenburg, R. L., Garner, G. B. 1986. Relationship between insect abundance and endophyte infection level in tall fescue in Missouri. *J. Kans. Entomol. Soc.* 59:552-54.
63. Kulkarni, R. K., Nielsen, B. D. 1986. Nutritional requirements for growth of a fungus endophyte of tall fescue. *Mycologia* 78:781-86.
64. Latch, G. C. M. 1983. Incidence of endophytes in seedlings and their control with fungicides. *Proc. N. Z. Grassl. Assoc.* 44:251-53.
65. Latch, G. C. M., Christensen, M. J. 1982. Ryegrass endophyte, incidence, and control. *N. Z. J. Agric. Res.* 25:443-48.
66. Latch, G. C. M., Christensen, M. J. 1985. Artificial infection of grasses with endophytes. *Ann. Appl. Biol.* 107:17-25.
67. Latch, G. C. M., Christensen, M. J. 1987. Endophytes of annual and hybrid ryegrasses. *N. Z. J. Agric. Res.* In press.
68. Latch, G. C. M., Christensen, M. J., Gaymer, D. L. 1985. Aphid detection of endophyte infection in tall fescue. *N. Z. J. Agric. Res.* 28:129-32.
69. Latch, G. C. M., Christensen, M. J., Samuels, G. J. 1984. Five endophytes of

- Johnson, M. C., Rust, J. P., Siegel, M. 1986. Infection of tall fescue with *Acremonium coenophialense* by means of dilute culture. *Plant Dis.* 70:98-99.
- Johnson, M. C., Daifotis, D. J., Siegel, M. R., Rust, J. P., Latch, G. M., et al. 1985. Insect feeding of termites in endophyte-infected tall fescue. *Appl. Environ. Microbiol.* 49:968-970.
- Johnson, M. C., Pringle, J. P., Siegel, M. R., Venzon, J. E. 1982. Detection of *Lopholium lyphina* in tall fescue by means of the thin layer chromatography assay. *Phytopathology* 72, 641-643.
- Johnson, M. C., Siegel, M. R., Schmidt, B. A. 1985. Serological analysis of endophyte fungi in tall fescue and perennial ryegrass and of *Epichloa typhina*. *Plant Dis.* 69:249-252.
- Jones, T. A., Buckner, R. C., Barrus, S. B., Bush, L. P. 1983. Accumulation of pyrrolizidine alkaloids in the ovine-treated tall fescue parents and non-treated progenies. *Crop Sci.* 23:135-40.
- Kennedy, C. W., Bush, L. P. 1983. Effect of environmental and management factors on the accumulation of N-cetyl and N-formyl leucine alkaloids in tall fescue. *Crop Sci.* 23:547-52.
- Kirman, G. W., Brandenburg, R. L., Jarner, G. B. 1986. Relationship between insect abundance and endophyte infestation level in tall fescue in Missouri. *J. Kans. Entomol. Soc.* 59:252.
- Kulkarni, R. K., Nielsen, B. D. 1986. Nutritional requirements for growth of a fungus endophyte of tall fescue. *Mycologia* 78:781-80.
- Latch, G. C. M. 1983. Incidence of endophytes in seedlings and their control with fungicides. *Proc. N. Z. Grassl. Soc.* 44:251-53.
- Latch, G. C. M., Christensen, M. J. 1982. Ryegrass endophyte incidence, seed control. *N. Z. J. Agric. Res.* 5:443-48.
- Latch, G. C. M., Christensen, M. J. 1985. Artificial infection of grasses with endophytes. *Ann. Appl. Biol.* 107:17-4.
- Latch, G. C. M., Christensen, M. J. 1987. Endophytes of annual and hybrid ryegrasses. *N. Z. J. Agric. Res.* In press.
- Latch, G. C. M., Christensen, M. J., Raynor, D. L. 1985. Aphid detection of endophyte infection in tall fescue. *N. Z. Agric. Res.* 28:129-32.
- Latch, G. C. M., Christensen, M. J., Amuels, G. J. 1984. Five endophytes of grasses and legumes in New Zealand. *Mycologia* 70:545-54.
- Latch, G. C. M., Hunt, W. E., Musgrave, D. R. 1985. Endophytes of grasses affect growth of perennial ryegrass. *N. Z. J. Agric. Res.* 28:165-68.
- Latch, G. C. M., Porter, I. R., Tyler, B. J. 1987. Incidence of endophytes in seeds from collections of *Lolium* and *Festuca* species. *Ann. Appl. Biol.* In press.
- Lewis, D. H. 1977. Concepts in fungal nutrition and the origin of endophytes. *Biol. Rev.* 48:26-39.
- Lewis, D. H. 1974. Micro-organisms and plants: The evolution of parasitism and mutualism. *24th Symp. Soc. Gen. Microbiol.*, pp. 367-97. Leyland, Cambridge Univ. Press.
- Lindau, G. 1904. Über das Vorkommen des Pilzes des *Lolium bolleum* in allgäusischen Saaten. *Sitzungsber. K. Preuss. Akad. Wissen.*, pp. 1041-36.
- Lloyd, A. B. 1959. The endophytic fungus of perennial ryegrass. *N. Z. J. Agric. Res.* 2:1187-92.
- Luttrell, E. S., Bacon, C. W. 1977. Classification of *Mutinospora* in the Clavicipitaceae. *Can. J. Bot.* 55:2090-92.
- Lyons, P. C., Plattner, R. D., Bacon, C. W. 1986. Occurrence of peptide and cyclic ergot alkaloids in tall fescue. *Science* 232:487-89.
- Marras, P. G. 1978. Ergotism in cattle. *See Ref.* 35, pp. 145-51.
- McLennan, E. 1970. The endophyte fungus of *Lolium*. *Proc. R. Soc. Victoria* 32:252-301.
- Morgan Jones, G., Gams, W. 1982. Notes on Hyphomycetes, XLII. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloa typhina*: new taxa in one of two new sections of *Acremonium*. *Mycotaxon* 55:311-18.
- Mortimer, P. H., di Menna, M. E. 1985. Interactions of *Lolium* endophyte on pasture production and perennial ryegrass staggers disease. In *Trichothecenes and Other Mycotoxins* ed. J. Lacey, pp. 349-58. Chichester: New York Wiley, 574 pp.
- Musgrave, D. R. 1984. Detection of an endophyte fungus of *Lolium perenne* using enzyme-linked immunosorbent assay (ELISA). *N. Z. J. Agric. Res.* 27:283-88.
- Musgrave, D. R., Fletcher, I. R. 1986. Optimisation and characterisation of enzyme-linked immunosorbent assay (ELISA) for the detection of the *Acremonium loliae* endophyte in *Lolium perenne*. *N. Z. J. Agric. Res.* 29:137-26.
- Musgrave, D. R., Grice, T. A., Latch, G. C. M., Christensen, M. J. 1986. Purification and characterisation of the antigens of endophyte fungi isolated from *Lolium perenne* and *Festuca arundinacea* in New Zealand. *N. Z. J. Agric. Res.* 29:121-28.
- Nell, J. C. 1940. The endophyte of ryegrass-*Lolium perenne*. *N. Z. J. Technol. Ser. A* 21:280-91.
- Nesher, A. 1958. Ueber einen in den Frucht von *Lolium temulentum* L. vorkommenden Pilz. *Beiz. Pflanzkrankh.* 36:207-14.
- Peterson, A. J., Bass, J. J., Bryant, M. J. 1984. Decreased plasma testosterone concentrations and weight gain in young bulls affected by ryegrass staggers. *N. Z. Vet. J.* 32:36-37.
- Phalipson, M. N., Christey, M. C. 1986. The relationship of host and endophyte during flowering, seed formation, and germination of *Lolium perenne*. *N. Z. J. Sci.* 24:125-34.
- Porter, J. K., Bacon, C. W., Cutler, H. G., Atterdale, R. I., Robbins, J. D. 1985. In vitro auxin production by *Balanus epichloe*. *Phytochemistry* 24:1429-31.
- Porter, J. K., Bacon, C. W., Robbins, J. D. 1979. Ergotism, ergonovine, and chachocaine I from *Epichloa typhina*. *J. Agric. Food Chem.* 27:595-98.
- Porter, J. K., Bacon, C. W., Robbins, J. D., Betowski, D. 1981. Ergot alkaloid identification in Clavicipitaceae systematic fungi of pasture grasses. *J. Agric. Food Chem.* 29:653-58.
- Puttinger, R. P., Baker, G. M., Prestidge, R. A. 1985. A review of the relationships between endophytic fungi of grasses (*Acremonium* spp.) and Argentine stem weevil (*Listronotus bonariensis* Kaschell). *Proc. 4th Australas. Conf. Grassl. Invert. Ecol. Lincoln Coll., Canterbury*, pp. 322-31.
- Prestidge, R. A., di Menna, M. E., van der Zyp, S. 1985. Minimum *Acremonium loliae* levels needed to maintain Argentine stem weevil resistance in infected ryegrasses and its cultures. *See Ref.* 82, pp. 332-37.
- Prestidge, R. A., Gallagher, R. T. 1985. Lolitrem B-A stem weevil toxin isolated from *Acremonium*-infected ryegrass. *Proc. N. Z. Weed Pest Control Conf.* 38:38-40.
- Prestidge, R. A., Lauren, D. R., van der Zyp, S. G., di Menna, M. E. 1985. Isolation of feeding deterrents to Argentine stem weevil in cultures of en-

86. Presavage, R. A., Pfallinger, R. P., Barakat, G. M. 1982. An association of *Lolium* endophyte with ryegrass toxicity to Argentine stem weevil. *Proc. N. Z. Weed Pest Control Conf.* 35: 13-27.
87. Reau, J. L., Camp, P. J. 1986. The effect of the fungal endophyte *Acremonium coenophialum* in tall fescue on animal performance, toxicity and stoma maintenance. *Agrostol* 2: 349-51.
88. Reuter, M. P., Hare, M. D., Moser, K. K., Christensen, M. J. 1986. *Lolium* endophyte fungi viability with storage at different seed moisture and temperatures. *N. Z. J. Exp. Agric.* 14: 297-300.
89. Rowan, D. D., Gayton, D. L. 1986. Isolation of feeding deterrents against Argentine stem weevil from ryegrass infected with the endophyte *Acremonium loliae*. *J. Chem. Ecol.* 12: 667-88.
90. Rowan, D. D., Hunt, M. B., Gayton, D. L. 1986. Peramine: a novel insect feeding deterrent from ryegrass infected with the endophyte *Acremonium loliae*. *J. Chem. Soc. Chem. Commun.* 955-56.
91. Ryland, D. M., Bacon, C. W., Lattrell, E. S. 1985. Host relations of *Mastigospora atramentosa* and *Balanus epiphyticus* (Clavicipitaceae). *Phytopathology* 75: 950-56.
92. Sampson, K. 1933. The systemic infection of grasses by *Euphorbia yspina* (Pers.) Tul. *Trans. Br. Mycol. Soc.* 18: 30-47.
93. Schmidt, S. P., Hoveland, C. S., Clark, E. M., Davis, N. D., Smith, L. A., et al. 1982. Association of an endophytic fungus with fescue toxicity in steers fed Kentucky 31 tall fescue seed or hay. *J. Anim. Sci.* 55: 1259-63.
94. Scott, D. J. 1983. *Lolium* endophyte levels in commercial perennial ryegrass seed lines. *Proc. N. Z. Grassl. Assoc.* 44: 254-56.
95. Shelby, R. A., Dalrymple, L. W. 1987. Incidence and distribution of the tall fescue endophyte in the United States. *Plant Dis. Rep.* in press.
96. Siegel, M. R., Johnson, M. C., Varney, D. R., Nesmith, W. C., Buckner, R. C., et al. 1984. A fungal endophyte in tall fescue: incidence and dissemination. *Phytopathology* 74: 932-37.
97. Siegel, M. R., Latch, G. C. M., Johnson, M. C. 1985. *Acremonium* fungal endophytes of tall fescue are perennial ryegrass: significance and control. *Plant Dis.* 69: 179-83.
98. Siegel, M. R., Varney, D. R., Johnson, M. C., Nesmith, W. C., Buckner, R. C., et al. 1984. A fungal endophyte of tall fescue. Evaluation of control methods. *Phytopathology* 74: 937-41.
99. Smith, K. T., Bacon, C. W., Lattrell, E. S. 1985. Reciprocal translocation of carbohydrates between host and fungus in bahiagrass infected with *Mastigospora atramentosa*. *Phytopathology* 75: 407-11.
100. Snell, W. Jr., Dick, E. A. 1971. *A Giesburt's Monograph*. Cambridge, Mass. Harvard Univ. Press.
101. Stewart, A. V. 1985. Perennial ryegrass seedling resistance to Argentine stem weevil. *N. Z. J. Agric. Res.* 78: 403-7.
102. Strahan, S. R., Henken, R. W., Jackson, J. A. Jr., Buckner, R. C., Bush, L. P., Siegel, M. R. 1987. Performance of lactating dairy cows fed tall fescue forage. *J. Dairy Sci.* In press.
103. Stuedemann, J. A., Runney, T. S., Bond, J., Wilkinson, S. R., Bush, L. P., et al. 1985. Association of blood cholesterol with occurrence of fat necrosis in cows and tall fescue summer toxicosis in steers. *Am. J. Vet. Res.* 46: 1998-95.
104. Varney, D. R., Ndefu, M., Jones, S. L., Siegel, M. R., Zavos, P. 1986. The effect of the fescue endophyte on the fat content of ewes. *Asian. Southeastern Biol. Bull.* 33: 66 (Abstr.).
105. Vogl, A. E. 1898. Mehl und die anderen Mehlprodukte der Cerealien und Leguminosen. *Nahrungsmittel Hyg. Warenk.* 12: 25-29.
106. Walker, J. C. 1950. *Plant Pathology*. New York: McGraw-Hill.
107. Wallner, B. M., Booth, N. H., Robbins, J. D., Bacon, C. W., Porter, J. K., et al. 1983. Effect of an endophytic fungus isolated from toxic pasture grass on serum prolactin concentrations in the lactating cow. *Am. J. Vet. Res.* 44: 1317-22.
108. Welty, R. E., Azevedo, M. D., Cook, K. L. 1986. Detecting viable *Acremonium* endophytes in leaf sheaths and meristems of tall fescue and perennial ryegrass. *Plant Dis.* 70: 431-35.
109. Welty, R. E., Milbrath, G. M., Faulkenberry, D., Azevedo, M. D., Meek, L., Hall, K. 1986. Endophyte detection in tall fescue seed by staining and ELISA. *Seed Sci. Technol.* 14: 105-16.
110. Western, J. H., Cavett, J. J. 1959. The choke disease of cocksfoot (*Dactylis glomerata*) caused by *Epicoccium typhina* (Fr.) Tul. *Trans. Br. Mycol. Soc.* 42: 298-307.
111. White, J. F. Jr. 1987. The widespread

1984. A fungal endophyte of sorghum. Evaluation of control. *Phytopathology* 4:93-401.
10. White, J. F. Jr., Cole, G. T., and Jones, G. 1987. Reciprocal translocation of isolates between host and fungus grass infected with *Acremonium coenophialum*. *Phytopathology* 78:28-30.
11. Dijk, L. A. 1972. *A Grasses*. Mycology. Cambridge, Mass: Univ. Press.
12. A. V. 1985. Perennial ryegrass resistance to Argentine stem weevil. *J. Agric. Res.* 28:105-106.
13. S. B., Henken, R. W., Jack, J. D., Buckner, R. C., Bush, I. J., and M. R. 1987. Performance of dairy cows fed tall fescue. *J. Dairy Sci.* In press.
14. Williams, M. J., Backman, P. A., and M. R. 1987. Performance of dairy cows fed tall fescue with occurrence of tall fescue toxicosis and tall fescue summer toxicosis. *Am. J. Vet. Res.* 48:95-97.
15. D. K., Niderm, M., Jones, G., and M. R., Zavos, P. 1986. The tall fescue endophyte on the rat cycle. *Assoc. Southeastern Res.* 66:40-41.
16. E. 1988. Mehl und die anderen dichte der Cerealia und Leguminosum. *Unters. Hye* 12:25-29.
17. J. C. 1950. *Plant Pathology*. McGraw-Hill.
18. B. M., Booth, N. H., Robbins, J. C., W., Porter, J. K., et al. 1985. Association of blood cell counts and endophytic fungus from toxic pasture grass on prolactin concentrations in the cow. *Am. J. Vet. Res.* 44:12-25.
19. K. E., Azevedo, M. D., Cook, 1986. Detecting viable *Acremonium* endophytes in leaf sheaths and roots of tall fescue and perennial ryegrass. *Plant Dis.* 70:431-435.
20. C. E., Milbrath, G. M., Faulkner, Azevedo, M. D., Meek, J. K. 1986. Endophyte detection in fescue seed by staining and seed. *Seed Sci. Technol.* 14:105-107.
21. J. H., Cavett, J. J. 1959. The disease of cocksfoot (*Dactylis glomerata*) caused by *Epichloa typhina*. *J. Trans. Br. Mycol. Soc.* 107:1-10.
22. J. F. 1987. The widespread distribution of endophytes in the Poaceae. *Plant Dis.* In press.
23. White, J. F. Jr., Cole, G. T. 1985. Endophyte host associations in forage grasses. I. Distribution of fungal endophytes in some species of *Lolium* and *Festuca*. *Mycologia* 77:323-327.
24. White, J. F. Jr., Cole, G. T. 1985. Endophyte host associations in forage grasses. III. In vitro inhibition of fungi by *Acremonium coenophialum*. *Mycologia* 77:487-494.
25. White, J. F. Jr., Cole, G. T. 1986. Endophyte host associations in forage grasses. IV. The endophyte of *Festuca ovina*. *Mycologia* 78:102-107.
26. White, J. F. Jr., Cole, G. T. 1986. Endophyte host associations in forage grasses. V. Occurrence of fungal endophytes in certain species of *Brachiaria* and *Poa*. *Mycologia* 78:846-850.
27. White, J. F. Jr., Cole, G. T., Morgan-Jones, G. 1987. Endophyte-host associations in forage grasses. VI. A new species of *Acremonium* isolated from *Festuca arizonica*. *Mycologia* 79:148-152.
28. White, J. F. Jr., Morgan-Jones, G. 1987. Endophyte-host associations in forage grasses. VII. *Acremonium chlamydosporum* a new species isolated from *Syntherisma* in Texas. *Mycologia* 78:179-185.
29. Williams, M. J., Backman, P. A., Clark, E. M., White, J. F. 1984. Seed treatments for control of the tall fescue endophyte *Acremonium coenophialum*. *Plant Dis.* 68:49-51.
30. Williams, M. J., Backman, P. A., Crawford, M. A., Schmidt, S. P., King, C. C. 1984. Chemical control of the tall fescue endophyte and its relationship to cattle performance. *N. Z. J. Exp. Agric.* 32:165-171.
31. Yates, S. G., Plattner, R. D., Garner, G. R. 1985. Detection of streptolysin alkaloids in endophyte infected toxic Ky 31 tall fescue by mass spectrometry. *J. Agric. Toxicol. Chem.* 33:719-722.
32. Zavos, P. M., Salari, B., Jackson, J. A., Varney, D. R., Siegel, M. R., Henken, R. W. 1986. Effect of feeding tall fescue seed infected by endophyte fungus (*Acremonium coenophialum*) on reproductive performance in male rats. *Theriogenology* 25:281-290.
33. Zavos, P. M., Varney, D. R., Jackson, J. A., Siegel, M. R., Bush, I. J., et al. 1987. Effect of feeding fungal endophyte (*Acremonium coenophialum*) infected tall fescue seed on reproductive performance in CD-1 mice through continuous breeding. *Theriogenology*. In press.
34. Zavos, P. M., Varney, D. R., Siegel, M. R., Henken, R. W., Jackson, J. A., et al. 1987. Effects of feeding endophyte-infected tall fescue seed on the reproductive performance in male and female CD-1 mice via combination crosses. *Theriogenology*. In press.

MYCOTAXON

Vol. XX, No. 2, pp. 535-550

July-September 1984

205

FIVE ENDOPHYTES OF *LOI LUM* AND *PESTICA* IN NEW ZEALAND ¹

G. C. M. LATCH ², M. J. CHIDSTER ², G. J. SAMBLES ²

Plant Diseases Division, DSIR, Private Bag,
Palmerston North ¹ and Auckland ², New Zealand

ABSTRACT

Five endophytic fungi were isolated from pasture grasses in New Zealand. *Epilobium*-like species and *Accumulium* *totale* Lich. (the former a new sp., nov. spec.) were isolated from *Loi lum*. A *Phialophora*-like species and *A. conchiforme* were isolated from *Pestica* and *Epilobium*. *Epilobium* Lich. (Persoon: Fr.) Tolmie was isolated from leaves and seed of apparently uninfected plants of *E. tubum* and from immature stems on seed heads of *E. tubum*. These endophytes are described and illustrated and their taxonomic relationships to previously reported but unidentified fungal endophytes of *Loi lum* and *Pestica* are discussed.

INTRODUCTION

Details of the morphology and distribution of endophytic fungi within seed and plants of pasture grasses have been known since the last century (Meldrum 1950, for a review) but only rarely have these endophytic fungi been identified or have sporulating structures been isolated. A number of endophytes have been identified on the basis of colony characteristics seen by microscopic examination (Simpson 1933, 1934, 1935, 1936, 1937, 1938, 1939, 1940, 1941, 1942, 1943, 1944, 1945, 1946, 1947, 1948, 1949, 1950, 1951, 1952, 1953, 1954, 1955, 1956, 1957, 1958, 1959, 1960, 1961, 1962, 1963, 1964, 1965, 1966, 1967, 1968, 1969, 1970, 1971, 1972, 1973, 1974, 1975, 1976, 1977, 1978, 1979, 1980, 1981, 1982, 1983, 1984, 1985, 1986, 1987, 1988, 1989, 1990, 1991, 1992, 1993, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005, 2006, 2007, 2008, 2009, 2010, 2011, 2012, 2013, 2014, 2015, 2016, 2017, 2018, 2019, 2020, 2021, 2022, 2023, 2024, 2025, 2026, 2027, 2028, 2029, 2030, 2031, 2032, 2033, 2034, 2035, 2036, 2037, 2038, 2039, 2040, 2041, 2042, 2043, 2044, 2045, 2046, 2047, 2048, 2049, 2050, 2051, 2052, 2053, 2054, 2055, 2056, 2057, 2058, 2059, 2060, 2061, 2062, 2063, 2064, 2065, 2066, 2067, 2068, 2069, 2070, 2071, 2072, 2073, 2074, 2075, 2076, 2077, 2078, 2079, 2080, 2081, 2082, 2083, 2084, 2085, 2086, 2087, 2088, 2089, 2090, 2091, 2092, 2093, 2094, 2095, 2096, 2097, 2098, 2099, 2100, 2101, 2102, 2103, 2104, 2105, 2106, 2107, 2108, 2109, 2110, 2111, 2112, 2113, 2114, 2115, 2116, 2117, 2118, 2119, 2120, 2121, 2122, 2123, 2124, 2125, 2126, 2127, 2128, 2129, 2130, 2131, 2132, 2133, 2134, 2135, 2136, 2137, 2138, 2139, 2140, 2141, 2142, 2143, 2144, 2145, 2146, 2147, 2148, 2149, 2150, 2151, 2152, 2153, 2154, 2155, 2156, 2157, 2158, 2159, 2160, 2161, 2162, 2163, 2164, 2165, 2166, 2167, 2168, 2169, 2170, 2171, 2172, 2173, 2174, 2175, 2176, 2177, 2178, 2179, 2180, 2181, 2182, 2183, 2184, 2185, 2186, 2187, 2188, 2189, 2190, 2191, 2192, 2193, 2194, 2195, 2196, 2197, 2198, 2199, 2200, 2201, 2202, 2203, 2204, 2205, 2206, 2207, 2208, 2209, 2210, 2211, 2212, 2213, 2214, 2215, 2216, 2217, 2218, 2219, 2220, 2221, 2222, 2223, 2224, 2225, 2226, 2227, 2228, 2229, 2230, 2231, 2232, 2233, 2234, 2235, 2236, 2237, 2238, 2239, 2240, 2241, 2242, 2243, 2244, 2245, 2246, 2247, 2248, 2249, 2250, 2251, 2252, 2253, 2254, 2255, 2256, 2257, 2258, 2259, 2260, 2261, 2262, 2263, 2264, 2265, 2266, 2267, 2268, 2269, 2270, 2271, 2272, 2273, 2274, 2275, 2276, 2277, 2278, 2279, 2280, 2281, 2282, 2283, 2284, 2285, 2286, 2287, 2288, 2289, 2290, 2291, 2292, 2293, 2294, 2295, 2296, 2297, 2298, 2299, 2300, 2301, 2302, 2303, 2304, 2305, 2306, 2307, 2308, 2309, 2310, 2311, 2312, 2313, 2314, 2315, 2316, 2317, 2318, 2319, 2320, 2321, 2322, 2323, 2324, 2325, 2326, 2327, 2328, 2329, 2330, 2331, 2332, 2333, 2334, 2335, 2336, 2337, 2338, 2339, 2340, 2341, 2342, 2343, 2344, 2345, 2346, 2347, 2348, 2349, 2350, 2351, 2352, 2353, 2354, 2355, 2356, 2357, 2358, 2359, 2360, 2361, 2362, 2363, 2364, 2365, 2366, 2367, 2368, 2369, 2370, 2371, 2372, 2373, 2374, 2375, 2376, 2377, 2378, 2379, 2380, 2381, 2382, 2383, 2384, 2385, 2386, 2387, 2388, 2389, 2390, 2391, 2392, 2393, 2394, 2395, 2396, 2397, 2398, 2399, 2400, 2401, 2402, 2403, 2404, 2405, 2406, 2407, 2408, 2409, 2410, 2411, 2412, 2413, 2414, 2415, 2416, 2417, 2418, 2419, 2420, 2421, 2422, 2423, 2424, 2425, 2426, 2427, 2428, 2429, 2430, 2431, 2432, 2433, 2434, 2435, 2436, 2437, 2438, 2439, 2440, 2441, 2442, 2443, 2444, 2445, 2446, 2447, 2448, 2449, 2450, 2451, 2452, 2453, 2454, 2455, 2456, 2457, 2458, 2459, 2460, 2461, 2462, 2463, 2464, 2465, 2466, 2467, 2468, 2469, 2470, 2471, 2472, 2473, 2474, 2475, 2476, 2477, 2478, 2479, 2480, 2481, 2482, 2483, 2484, 2485, 2486, 2487, 2488, 2489, 2490, 2491, 2492, 2493, 2494, 2495, 2496, 2497, 2498, 2499, 2500, 2501, 2502, 2503, 2504, 2505, 2506, 2507, 2508, 2509, 2510, 2511, 2512, 2513, 2514, 2515, 2516, 2517, 2518, 2519, 2520, 2521, 2522, 2523, 2524, 2525, 2526, 2527, 2528, 2529, 2530, 2531, 2532, 2533, 2534, 2535, 2536, 2537, 2538, 2539, 2540, 2541, 2542, 2543, 2544, 2545, 2546, 2547, 2548, 2549, 2550, 2551, 2552, 2553, 2554, 2555, 2556, 2557, 2558, 2559, 2560, 2561, 2562, 2563, 2564, 2565, 2566, 2567, 2568, 2569, 2570, 2571, 2572, 2573, 2574, 2575, 2576, 2577, 2578, 2579, 2580, 2581, 2582, 2583, 2584, 2585, 2586, 2587, 2588, 2589, 2590, 2591, 2592, 2593, 2594, 2595, 2596, 2597, 2598, 2599, 2600, 2601, 2602, 2603, 2604, 2605, 2606, 2607, 2608, 2609, 2610, 2611, 2612, 2613, 2614, 2615, 2616, 2617, 2618, 2619, 2620, 2621, 2622, 2623, 2624, 2625, 2626, 2627, 2628, 2629, 2630, 2631, 2632, 2633, 2634, 2635, 2636, 2637, 2638, 2639, 2640, 2641, 2642, 2643, 2644, 2645, 2646, 2647, 2648, 2649, 2650, 2651, 2652, 2653, 2654, 2655, 2656, 2657, 2658, 2659, 2660, 2661, 2662, 2663, 2664, 2665, 2666, 2667, 2668, 2669, 2670, 2671, 2672, 2673, 2674, 2675, 2676, 2677, 2678, 2679, 2680, 2681, 2682, 2683, 2684, 2685, 2686, 2687, 2688, 2689, 2690, 2691, 2692, 2693, 2694, 2695, 2696, 2697, 2698, 2699, 2700, 2701, 2702, 2703, 2704, 2705, 2706, 2707, 2708, 2709, 2710, 2711, 2712, 2713, 2714, 2715, 2716, 2717, 2718, 2719, 2720, 2721, 2722, 2723, 2724, 2725, 2726, 2727, 2728, 2729, 2730, 2731, 2732, 2733, 2734, 2735, 2736, 2737, 2738, 2739, 2740, 2741, 2742, 2743, 2744, 2745, 2746, 2747, 2748, 2749, 2750, 2751, 2752, 2753, 2754, 2755, 2756, 2757, 2758, 2759, 2760, 2761, 2762, 2763, 2764, 2765, 2766, 2767, 2768, 2769, 2770, 2771, 2772, 2773, 2774, 2775, 2776, 2777, 2778, 2779, 2780, 2781, 2782, 2783, 2784, 2785, 2786, 2787, 2788, 2789, 2790, 2791, 2792, 2793, 2794, 2795, 2796, 2797, 2798, 2799, 2800, 2801, 2802, 2803, 2804, 2805, 2806, 2807, 2808, 2809, 2810, 2811, 2812, 2813, 2814, 2815, 2816, 2817, 2818, 2819, 2820, 2821, 2822, 2823, 2824, 2825, 2826, 2827, 2828, 2829, 2830, 2831, 2832, 2833, 2834, 2835, 2836, 2837, 2838, 2839, 2840, 2841, 2842, 2843, 2844, 2845, 2846, 2847, 2848, 2849, 2850, 2851, 2852, 2853, 2854, 2855, 2856, 2857, 2858, 2859, 2860, 2861, 2862, 2863, 2864, 2865, 2866, 2867, 2868, 2869, 2870, 2871, 2872, 2873, 2874, 2875, 2876, 2877, 2878, 2879, 2880, 2881, 2882, 2883, 2884, 2885, 2886, 2887, 2888, 2889, 2890, 2891, 2892, 2893, 2894, 2895, 2896, 2897, 2898, 2899, 2900, 2901, 2902, 2903, 2904, 2905, 2906, 2907, 2908, 2909, 2910, 2911, 2912, 2913, 2914, 2915, 2916, 2917, 2918, 2919, 2920, 2921, 2922, 2923, 2924, 2925, 2926, 2927, 2928, 2929, 2930, 2931, 2932, 2933, 2934, 2935, 2936, 2937, 2938, 2939, 2940, 2941, 2942, 2943, 2944, 2945, 2946, 2947, 2948, 2949, 2950, 2951, 2952, 2953, 2954, 2955, 2956, 2957, 2958, 2959, 2960, 2961, 2962, 2963, 2964, 2965, 2966, 2967, 2968, 2969, 2970, 2971, 2972, 2973, 2974, 2975, 2976, 2977, 2978, 2979, 2980, 2981, 2982, 2983, 2984, 2985, 2986, 2987, 2988, 2989, 2990, 2991, 2992, 2993, 2994, 2995, 2996, 2997, 2998, 2999, 3000, 3001, 3002, 3003, 3004, 3005, 3006, 3007, 3008, 3009, 3010, 3011, 3012, 3013, 3014, 3015, 3016, 3017, 3018, 3019, 3020, 3021, 3022, 3023, 3024, 3025, 3026, 3027, 3028, 3029, 3030, 3031, 3032, 3033, 3034, 3035, 3036, 3037, 3038, 3039, 3040, 3041, 3042, 3043, 3044, 3045, 3046, 3047, 3048, 3049, 3050, 3051, 3052, 3053, 3054, 3055, 3056, 3057, 3058, 3059, 3060, 3061, 3062, 3063, 3064, 3065, 3066, 3067, 3068, 3069, 3070, 3071, 3072, 3073, 3074, 3075, 3076, 3077, 3078, 3079, 3080, 3081, 3082, 3083, 3084, 3085, 3086, 3087, 3088, 3089, 3090, 3091, 3092, 3093, 3094, 3095, 3096, 3097, 3098, 3099, 3100, 3101, 3102, 3103, 3104, 3105, 3106, 3107, 3108, 3109, 3110, 3111, 3112, 3113, 3114, 3115, 3116, 3117, 3118, 3119, 3120, 3121, 3122, 3123, 3124, 3125, 3126, 3127, 3128, 3129, 3130, 3131, 3132, 3133, 3134, 3135, 3136, 3137, 3138, 3139, 3140, 3141, 3142, 3143, 3144, 3145, 3146, 3147, 3148, 3149, 3150, 3151, 3152, 3153, 3154, 3155, 3156, 3157, 3158, 3159, 3160, 3161, 3162, 3163, 3164, 3165, 3166, 3167, 3168, 3169, 3170, 3171, 3172, 3173, 3174, 3175, 3176, 3177, 3178, 3179, 3180, 3181, 3182, 3183, 3184, 3185, 3186, 3187, 3188, 3189, 3190, 3191, 3192, 3193, 3194, 3195, 3196, 3197, 3198, 3199, 3200, 3201, 3202, 3203, 3204, 3205, 3206, 3207, 3208, 3209, 3210, 3211, 3212, 3213, 3214, 3215, 3216, 3217, 3218, 3219, 3220, 3221, 3222, 3223, 3224, 3225, 3226, 3227, 3228, 3229, 3230, 3231, 3232, 3233, 3234, 3235, 3236, 3237, 3238, 3239, 3240, 3241, 3242, 3243, 3244, 3245, 3246, 3247, 3248, 3249, 3250, 3251, 3252, 3253, 3254, 3255, 3256, 3257, 3258, 3259, 3260, 3261, 3262, 3263, 3264, 3265, 3266, 3267, 3268, 3269, 3270, 3271, 3272, 3273, 3274, 3275, 3276, 3277, 3278, 3279, 3280, 3281, 3282, 3283, 3284, 3285, 3286, 3287, 3288, 3289, 3290, 3291, 3292, 3293, 3294, 3295, 3296, 3297, 3298, 3299, 3300, 3301, 3302, 3303, 3304, 3305, 3306, 3307, 3308, 3309, 3310, 3311, 3312, 3313, 3314, 3315, 3316, 3317, 3318, 3319, 3320, 3321, 3322, 3323, 3324, 3325, 3326, 3327, 3328, 3329, 3330, 3331, 3332, 3333, 3334, 3335, 3336, 3337, 3338, 3339, 3340, 3341, 3342, 3343, 3344, 3345, 3346, 3347, 3348, 3349, 3350, 3351, 3352, 3353, 3354, 3355, 3356, 3357, 3358, 3359, 3360, 3361, 3362, 3363, 3364, 3365, 3366, 3367, 3368, 3369, 3370, 3371, 3372, 3373, 3374, 3375, 3376, 3377, 3378, 3379, 3380, 3381, 3382, 3383, 3384, 3385, 3386, 3387, 3388, 3389, 3390, 3391, 3392, 3393, 3394, 3395, 3396, 3397, 3398, 3399, 3400, 3401, 3402, 3403, 3404, 3405, 3406, 3407, 3408, 3409, 3410, 3411, 3412, 3413, 3414, 3415, 3416, 3417, 3418, 3419, 3420, 3421, 3422, 3423, 3424, 3425, 3426, 3427, 3428, 3429, 3430, 3431, 3432, 3433, 3434, 3435, 3436, 3437, 3438, 3439, 3440, 3441, 3442, 3443, 3444, 3445, 3446, 3447, 3448, 3449, 3450, 3451, 3452, 3453, 3454, 3455, 3456, 3457, 3458, 3459, 3460, 3461, 3462, 3463, 3464, 3465, 3466, 3467, 3468, 3469, 3470, 3471, 3472, 3473, 3474, 3475, 3476, 3477, 3478, 3479, 3480, 3481, 3482, 3483, 3484, 3485, 3486, 3487, 3488, 3489, 3490, 3491, 3492, 3493, 3494, 3495, 3496, 3497, 3498, 3499, 3500, 3501, 3502, 3503, 3504, 3505, 3506, 3507, 3508, 3509, 3510, 3511, 3512, 3513, 3514, 3515, 3516, 3517, 3518, 3519, 3520, 3521, 3522, 3523, 3524, 3525, 3526, 3527, 3528, 3529, 3530, 3531, 3532, 3533, 3534, 3535, 3536, 3537, 3538, 3539, 3540, 3541, 3542, 3543, 3544, 3545, 3546, 3547, 3548, 3549, 3550, 3551, 3552, 3553, 3554, 3555, 3556, 3557, 3558, 3559, 3560, 3561, 3562, 3563, 3564, 3565, 3566, 3567, 3568, 3569, 3570, 3571, 3572, 3573, 3574, 3575, 3576, 3577, 3578, 3579, 3580, 3581, 3582, 3583, 3584, 3585, 3586, 3587, 3588, 3589, 3590, 3591, 3592, 3593, 3594, 3595, 3596, 3597, 3598, 3599, 3600, 3601, 3602, 3603, 3604, 3605, 3606, 3607, 3608, 3609, 3610, 3611, 3612, 3613, 3614, 3615, 3616, 3617, 3618, 3619, 3620, 3621, 3622, 3623, 3624, 3625, 3626, 3627, 3628, 3629, 3630, 3631, 3632, 3633, 3634, 3635, 3636, 3637, 3638, 3639, 3640, 3641, 3642, 3643, 3644, 3645, 3646, 3647, 3648, 3649, 3650, 3651, 3652, 3653, 3654, 3655, 3656, 3657, 3658, 3659, 3660, 3661, 3662, 3663, 3664, 3665, 3666, 3667, 3668, 3669, 3670, 3671, 3672, 3673, 3674, 3675, 3676, 3677, 3678, 3679, 3680, 3681, 3682, 3683, 3684, 3685, 3686, 3687, 3688, 3689, 3690, 3691, 3692, 3693, 3694, 3695, 3696, 3697, 3698, 3699, 3700, 3701, 3702, 3703, 3704, 3705, 3706, 3707, 3708, 3709, 3710, 3711, 3712, 3713, 3714, 3715, 3716, 3717, 3718, 3719, 3720, 3721, 3722, 3723, 3724, 3725, 3726, 3727, 3728, 3729, 3730, 3731, 3732, 3733, 3734, 3735, 3736, 3737, 3738, 3739, 3740, 3741, 3742, 3743, 3744, 3745, 3746, 3747, 3748, 3749, 3750, 3751, 3752, 3753, 3754, 3755, 3756, 3757, 3758, 3759, 3760, 3761, 3762, 3763, 3764, 3765, 3766, 3767, 3768, 3769, 3770, 3771, 3772, 3773, 3774, 3775, 3776, 3777, 3778, 3779, 3780, 3781, 3782, 3783, 3784, 3785, 3786, 3787, 3788, 3789, 3790, 3791, 3792, 3793, 3794, 3795, 3796, 3797, 3798, 3799, 3800, 3801, 3802, 3803, 3804, 3805, 3806, 3807, 3808, 3809, 3810, 3811, 3812, 3813, 3814, 3815, 3816, 3817, 3818, 3819, 3820, 3821, 3822, 3823, 3824, 3825, 3826, 3827, 3828, 3829, 3830, 3831, 3832, 3833, 3834, 3835, 3836, 3837, 3838, 3839, 3840, 3841, 3842, 3843, 3844, 3845, 3846, 3847, 3848, 3849, 3850, 3851, 3852

In culture as Lelli persona, Calicut, North Malabar, New Zealand, G.C.M. Latch et al. (Christensen & 20, 2 Sep 1981, (PUB 44, 17).

Columns from Live Wrecks on Playa (1991)

Colony size, conventional, aerial mycelium lacking, colony aerial (Table 1). Colonies arose on PDA and OA media. First, waxy, with or without spores, white aerial hyphae on the surface of some cultures on OA media. Aerial hyphae, the medium, flat or beaking, aerial mycelium, then sparsely, consisting of scattered, erect hyphae, less frequently, scanty and loosely entangling; hyaline 1.5–2.0 (2–2.5) μm wide. *Conidiophores* arising dichotomically in short, only from aerial tips abundantly, multiseptate, branched, branched, branched, branching point from 1.5–2.0 μm wide at base to 1.0–1.5 μm wide at tip; (8–10) 13.5–16.5 (10–11) μm long, lacking a basal septum, rarely 1.2–2-septate, wall thin, smooth, thin, the as spores in the subabundant, hyaline, oval, ellipsoid, 2-septate, (7–10) \times (1.5–2) 1.5–2 (1–1.5) μm , thick, smooth, (7–10) \times (1.5–2) 1.5–2 (1–1.5) μm , thick, smooth, $M_n = 6.6$, $SD = 1.3$ μm ; $M_w = 2.2$, $SD = 0.3$ μm) unilocular, bilocular.

TABLE 1. Growth of *Epithelium* c., *Leishmania* *Acetabulum* *solana* and *A. voronchikhini* measured after 3 and 5 weeks in darkness.

| Temperature ($^{\circ}\text{C}$) | Column diameter ^a (mm) | Flow rate (mL/min) |
|------------------------------------|--------------------------------------|-----------------------|
| 0 | 4 | 5 |
| | 10 | 5 |
| | 15 | 5 |
| | 20 | 5 |
| | 25 | 5 |

| CT | α/α^2 | α/α^2 | α/α^2 |
|------|-------------------|-------------------|-------------------|
| GA | 10/20 | 9/19 | 10/25 |
| PI/A | 12/17 | 10/15 | 12/17 |
| CM/B | 12/20 | 11/17 | 13/21 |

[illegible]

the quantity of nodules of the stroma.

Stroma (Fig. 1) of cells of nodules, separately (2-3) per cell, is located in the 2-layered outer surface of the nodules and in the extension of underlying hyphae. A thin layer of nodules is constituted the terminal 1-2 layers of the hyphae. The nodules are located in the stroma, which appears as found in culture (Fig. 1), and are observed in our material. Nodules are located in the stroma (Fig. 1, 2). Stroma, 3-4 μ m in diameter, is located in the base of conidiophores or in septate hyphae of stroma hyphae, they were separated from the nodules by the use of a special method. The nodules are located in the stroma hyphae, they were separated from the stroma hyphae (Fig. 1, 2) for E. Lipidum.

Examination of nodules of E. Lipidum

Microscopic (Fig. 1, 2) 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Examination of nodules of E. Lipidum, 1982. Page 1, 2.

Page 2, 3.

Page 2, 3.

Page 2, 3.

Page 2, 3.

Page 2, 3.

Page 2, 3.

Page 2, 3.

Page 2, 3.

Page 2, 3.

Page 2, 3.



FIG. 2. E. Lipidum-like nodules of E. Lipidum.

A. nodules of E. Lipidum (10 μ m scale bar).

B. nodules of E. Lipidum (10 μ m scale bar).

C. nodules of E. Lipidum (10 μ m scale bar).

D. nodules of E. Lipidum (10 μ m scale bar).

E. Lipidum-like nodules of E. Lipidum.

New York, New York: New York University Press, 1997. Pp. 288. \$35.00. ISBN 0 8147 2500 0.

[illegible]
$$v_i = (v_{i1}, \dots, v_{iN}) \text{ and } v_i \in \mathbb{R}^N \text{ for } i = 1, \dots, N. \text{ Let } v = (v_1, \dots, v_N) \text{ and } v \in \mathbb{R}^{N \times N}. \text{ Let } v_i = (v_{i1}, \dots, v_{iN}) \text{ and } v_i \in \mathbb{R}^N \text{ for } i = 1, \dots, N. \text{ Let } v = (v_1, \dots, v_N) \text{ and } v \in \mathbb{R}^{N \times N}.$$
[illegible][illegible][illegible]

sculptonophyllous *Evallivum* and *Clavus* are distinguished from the same basic morphology and therefore seem to be convergent. The form of these anamorphs in nature is comparable to the shell-like anamorph of a third-genitator-like member of the clavicipitales, *Hyphomycetella* (1982) (Johansen 1984, p. 449). In brief, the members of the clavicipitales form very complex conidia that conform to the contour of the presporel stroma. The same is observed in other non-teleomorphic ascomycetes. Perhaps of *Nectria* (*Strobilomyces*) (*Pohl* + *Pohl*) *Pico* form on the surface of the apothecium of *Telebasium* *valdeolus* (not after the cessation of conical projection from within) in pure form. *Evallivum* and *Strobilomyces* are known to give birth to *Evallivum* and *Strobilomyces* that degenerate into stromatolite, and in *Hyphomycetella* *degeneratilis* and other members of the *Evallivum* (*Pohl* 1990) conidia form hyaline aggregates and non-degenerated conidiophores form in agar culture. Separate generic names are not given to the multilayered expressions of the *Evallivum* or *Nectria* and we do not believe that the critical separation of *Evallivum* and *Nectria* warrants a separate name in accordance with the International Code of Nomenclature for Fungi. We therefore propose the name of *Evallivum* (*Pohl* 1990). In the absence of a critical review of taxonomy of the anamorph genera attributed to the *Evallivum* and *Clavicipitales*, we prefer to refer the anamorph of *Evallivum* to *Strobilomyces*.

Conidia and conidiophores of *Ascremium* (*Ascremium* and *A. fallax*) are so strikingly similar to those of the *Ascremium*-like conidiophores found in cultures of *E. coli*. Lyellian suggests that there may be three species of *Ascremium* present in New Zealand pasture flowers and that the species in *Ascremium* sect. *Ascremium* may comprise the two species in *Ascremium* sect. *Ascremium*. Notopoulos & Gams (1962) on the basis of morphology of the conidiophore alone and in the belief that they are *Ascremium* species, have previously attributed to other *Ascremium* species. Because perfect identification is not seen in the tip of the conidiogenous cell and because only one conidium is borne on each conidiogenous cell, it is possible that conidiogenesis in these three species is holoblastic, thus separating them further from other *Ascremium* species. *Ascremium* (*Ascremium*) *fallax* should be placed in a separate genus, possibly named by specialists in mycological taxonomy, and conidiogenesis in *Ascremium* (*Ascremium*) *fallax* is holoblastic but the conidiogenous locus proliferates sympatrically after the formation of each conidium (Rehner et al., 1962).

The Glaciolacian and Placatophora entophytes.

The Glycolobium-like and Phyllophora-like entophytes described in this paper are very generalized in morphology. Their generic names reflect that morphology without implying phylogenetic relationship to other species of the genera. We refer to the parietal endophyte as 'beard'.

